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Preparation of Extracts of Urine and Organs for Estimation of Free and Conjugated Noradrenaline and Adrenaline.

By

U. S. VON EULER and I. ORWÉN.

In most extracts of biological origin the catechol amines adrenaline and noradrenaline occur together with a variety of other substances which may interfere seriously with the assay, whether it be carried out by biological or chemical methods. The introduction of the method of adsorption on aluminium hydroxide by SHAW (1938) represented a useful means of obtaining an almost specific adsorption of catechol compounds. If aluminium hydroxide is formed in the solution from aluminium sulfate and sodium hydroxide at pH 7.6—7.8 (EULER, 1948), smaller amounts are needed for complete adsorption. After dissolving in acid the aluminium salts can be removed by precipitation with an ethanol-acetone mixture. This method yields extracts suitable for bioassay and also for chemical (colorimetric, fluorimetric) estimation. The method requires many steps of preparation, however, and an attempt was therefore made to simplify and shorten the procedure. The present report describes a modification of the method, using adsorption on aluminium oxide at pH 8.5, and subsequent elution with acid. Similar methods have been used by LUND (1952) and PEKKARINEN (1954).

I. Urine.

After administration of catechol amines part of them are excreted in urine in conjugated form (RICHTER, 1940). Normal urine also contains a mixture of catechol amines, partly in free

form, partly conjugated (HOLTZ, CREDNER and KRONEBERG, 1947). In human urine the presence of noradrenaline, adrenaline, and dopamine has been demonstrated by various methods (EULER, HAMBERG and HELLNER, 1951).

The present method has been worked out and tested for free as well as for total catechol amines.

The influence of several methodological factors has been tested by adding catechol amines to the urine before extraction and determining the recovery.

The catechol amines were estimated by biological assay according to the method of EULER (1949). The standard deviation of the recovery figures obviously includes the errors involved in the biological assay, which may be estimated at $\pm$ 10 p.c. for blood pressure determinations (noradrenaline) and $\pm$ 20 p.c. for chicken rectum assay (adrenaline).

a) *Amount of aluminium oxide.*

Preliminary experiments with passage of urine through a column of aluminium oxide proved unsatisfactory. Better results were obtained by continuous stirring of the urine with aluminium oxide at pH 8.5 by means of a magnetic stirrer. In order to ensure optimal adsorption of the catechol amines the amount of aluminium oxide must be above a certain minimum. On the other hand, large amounts lead to unfavourable elution conditions and also offer other disadvantages. By studying the recovery of noradrenaline and adrenaline in aqueous solutions or after addition to urine before the procedure, it was found that 2.5 g aluminium oxide was a suitable quantity when 100 ml urine of average concentration (1—1.5 litres per 24 hours) was used.

Table I.

Recovery in per cent of added noradrenaline to urine with various amounts of Al_2O_3 and elution with 2 × 5 ml 0.25 N sulfuric acid.

Al_2O_3 g/100 ml urine	Noradr. added μg	Recovery in eluate % of added	Recovery in filtrate % of added
0.2	10	—	100
0.5	»	7	65
1.0	»	65, 75	—
1.5	»	56, 75	—
2.0	»	65, 67	< 20
2.5	»	64, 75, 80, 85	—
3.0	»	90	—
5.0	»	70, 75	< 10
10	»	80	< 5

b) *Adsorption pH.*

It has been stated by LUND (1949) and by PEKKARINEN (1954) that adsorption of catechol amines is optimal at pH 8.5. Above this pH the catechol amines are liable to become oxidized and inactivated. By the present technique where the aluminium oxide is added to the solution before alkalization and the reaction subsequently adjusted to pH 8.5, the risk of inactivation is diminished. The following Table (II) shows the influence of various pH values on the recovery of noradrenaline added to urine. It was essential, however, to add the aluminium oxide before alkalization; if alkali was added first to pH 8.5, losses of up to 30 p.c. were observed.

Table II.

Effect of various pH on adsorption of noradrenaline added to urine.

Final pH in aluminium oxide suspension	Recovery in % of added noradrenaline (10 µg/100 ml urine)
7.0	13
8.0	55
8.3	70, 65
8.5	80, 75
8.7	80, 70
9.0	67

The recovery was not diminished if the solution was left with aluminium oxide at pH 8.5 for 15 min. After 30 min. the loss was still less than 25 %, but after 75 min. a loss of 33 % of the activity was noted.

Addition of thiosulfate did not have any noticeable effect on the recovery from urine, while addition of NaF to 0.25 % diminished the yield. If the reaction was adjusted to pH 8.5 with Na_2CO_3 instead of with NaOH the recovery was smaller and even less so after stirring, which improved the recovery with NaOH.

c) *Elution.*

In order to obtain eluates suitable for biologic assay, sulfuric acid was used for elution of the catechol amines (U. HAMBERG). It was found essential that the eluate should have a pH below 4. Table III shows the effect of various concentrations of sulfuric acid on the recovery. The recovery after one elution with 5 ml 0.25 N-H_2SO_4 was usually below 50 %. For practical reasons the amounts used were 2×5 ml (2×4 ml). Smaller amounts were insufficient to ensure a complete mixing and larger amounts

Table III.

Concentration of sulfuric acid (2 × 5 ml)	Recovery in % of added noradrenaline (10 μg/100 ml urine)
0.1 N	< 50
0.15 »	100
0.2 »	80—100
0.3 »	75
0.4 »	60—100

were often unsuitable for the assay. Optimal recovery was obtained even at an elution time of 1 min.

With amounts of aluminium oxide larger than 5 g more acid was usually needed for the elution. Satisfactory results were also obtained by elution with 0.5 N oxalic acid, as used by PEKKARINEN.

Final Procedure.

a) *Free catechol amines in urine.*

For the preparation of the free catechol amines in urine a sample corresponding to about 2 hours excretion (usually 50—200 ml) is used. After adjustment to pH 3 with HCl, the urine is heated to boiling and filtered if necessary. It is then transferred to a 250—500 ml beaker and 2.5 g aluminium oxide for chromatographic use added. During continuous mixing with a magnetic stirrer 0.5 N-NaOH is added dropwise until the pH is around 7. This point is conveniently noted if 0.05 ml 1 % bromthymol-blue has previously been added to the urine. Further sodium hydroxide is added until a pH of 8.5 is reached as estimated with thymol-blue pH-paper. After sedimentation the suspension is filtered on a suction funnel of 5 cm diameter. Two sheets of filter paper Munktell 1 F have been found to give a suitable filtration speed. The aluminium oxide layer is washed twice with 10—20 ml distilled water and sucked moist dry. The filtrate is discarded.

The Büchner funnel is then transferred to a small suction flask and 5 ml 0.25 N sulfuric acid added. After thorough mixing with the aluminium oxide mild suction is applied until the eluate has filtered through. The procedure is repeated once with 4 ml of the sulfuric acid. To the acid eluate is added 0.05 ml 1 % bromphenol-blue and the reaction adjusted with 10 % sodium bicarbonate

to pH 4. The volume is then made up to 10 ml with distilled water in a graduated cylinder.

When assayed on biological preparations the extracts display the typical actions of the catechol amines. On rare occasions urine may contain large amounts of histamine of which a small proportion may occur in the extract. By using a suitable anti-histaminic (phenergan) the histamine does not interfere with the biological assay.

Recovery of added catechol amines.

The following Table (IV) shows the recovery in % of the added amounts when the final procedure was adopted.

Table IV.

Noradrenaline added per 100 ml urine	Recovery in %
1	100
2	100
3	67
10	70—90
15	80
20	75—85
40	65
100	77
aq.dest.	
200	82

From Table IV it can be seen that the recovery is about 80 % on an average when the amounts added are in the range of 1—100 μg per 100 ml. The relative recovery is apparently independent of the amounts added within these limits. The usual range of noradrenaline content in urine is 1—10 μg noradrenaline per 100 ml but may occasionally rise to 100—200 μg per 100 ml in cases of phaeochromocytoma. In 27 estimations the recovery after addition of 10 μg noradrenaline to 100 ml was 78 % (S.D. 9.5). In 10 estimations the recovery of adrenaline was 85 % (S.D. 11). When the urine excretion exceeded 100 ml per hour 200 ml urine may be used. An increase of the aluminium oxide amount to 5 g is then preferable.

If the urine volume excreted in one or two hours is small, it is conveniently diluted to 100 ml with distilled water before adsorption. Some urine samples have a tendency to filter slowly

after adsorption. The filtration may be improved by adding 0.5 g Hyflo Super Cel to 100 ml urine at pH 3—4. Addition of larger amounts tend to decrease the yield.

When elution is made with 0.5 N oxalic acid the extracts have been found suitable for fluorimetric estimation according to EULER and FLODING (1955 a, b).

b) *Conjugated catechol amines.*

A series of experiments were made in order to ascertain the conditions for hydrolysis of conjugated catechol amines. The urine was adjusted with sulfuric acid to different pH-values and heated for varying lengths of time at 100°C. The pH was repeatedly checked and adjusted if necessary during the heating.

Table V shows the effect of heating for 20 min. at different pH-values on the recovery of noradrenaline in urine samples. Similar figures were found for adrenaline.

Table V.

Effect of heating urine for 20 min. at 100°C at varying pH on recovery of added noradrenaline.

pH	Per cent recovered of added 10 μg noradrenaline per 100 ml			
	Expt.			Average
	1	2	3	
3.0	90	80	90	87
2.0	70	80	95	82
1.5	70	70	100	80
1.0	65	75	90	77
0	80		90	85

The effect on the yield of catechol amines caused by heating urine for 20 min. at various pH-values is given in Table VI.

From Table VI it can be concluded that at pH 2 or higher no appreciable splitting of conjugated compounds takes place. The figures obtained after hydrolysis at pH 0 are doubled due to the complete racemization which occurs during this treatment and reduces the biological estimate to one half (Table V and VI).

At a pH of approximately 0 (11 volumes 10 N-HCl to 100 ml urine) the urine was heated for 10—60 minutes and the catechol amine content assayed. No difference in activity between these samples could be detected. It may therefore be concluded that splitting of conjugated compounds and racemization is completed

Table VI.

Effect of heating urine for 20 minutes at varying pH on the percentage yield of catechol amines compared with untreated urine (100 %).

pH	Range and average	
	Adrenaline	Noradrenaline
3	100	98, 100 (99)
2	100—128 (114)	105—115 (105)
1.5	138—156 (147)	108—139 (120)
1	122—131 (126)	98—157 (132)
0	206—217 (212)	115—258 (178)

in 10 minutes. This does not exclude the possibility that some other more stable conjugates are present which decompose more slowly as may be the case with glucuronides. For many purposes the procedure of heating with 11 volumes 10 N-HCl for 10 minutes is considered to give a satisfactory figure for the total catechol amines.

In a series of determinations from 100 urine specimens the catechol amines were increased 1—2 times in 54 %, 2—3 times in 27 %, and > 3 times in 19 % after acid hydrolysis at pH 0 for 20 minutes. The mean recovery in acid-treated urine after addition of 10 μg noradrenaline to 100 ml of urine before hydrolysis was in 19 experiments 81 % (S.D. = 9.9).

Stability of catechol amines in the eluate.

When stored at room temperature no inactivation was noted in the eluate (pH 4) after 48 hours. In the refrigerator (+ 3°C) the extracts maintained their full activity for a week and in the deep freeze box (— 23°C) no loss of activity was detected after a month. No systematic studies of the deterioration rate at different temperatures were made, however.

Specificity of adsorption.

Some experiments were made by adding phenyl and monohydroxyphenyl amines and then determining the recovery. Only a low percentage was recovered of ephedrine or tyramine in each case. These results agree with previous findings and statements that adsorption on aluminium oxide is apparently specific for dihydroxyphenyl compounds.

II. Preparation of Organ Extracts.

In preparing organ extracts for adsorption on aluminium oxide the procedure followed that described earlier (EULER, 1949). The organ was minced with an appropriate amount of trichloroacetic acid in a Waring blendor. Small organs of 1—2 g weight were ground with fine sand in a mortar with 5—10 volumes 5 % trichloroacetic acid. The concentrations of trichloroacetic acid necessary to ensure a clear filtrate depend on the volume of extraction fluid and on the type of organ. For a cat spleen weighing 5 g, 100 ml 5 % trichloroacetic acid is suitable, while for a liver weighing 100 g, 300 ml of a 5—10 % solution is usually adequate. After mincing, the tissue suspension is allowed to stand with occasional stirring for 1/4 hour and is then filtered on folded paper without suction. The residue is washed twice with 1/10 the original volume 5 % trichloroacetic acid. 2.5 g aluminium oxide is then added to the combined filtrates or to an aliquot having a volume of 100—200 ml and the procedure continued as described for urine.

For biological assay purposes it is often convenient to reduce the extract volume by evaporation at pH 3—4 at room temperature, preferably in vacuo.

The results obtained with the present adsorption technique compare well with those obtained with the previous more elaborate technique (EULER and UDDÉN, 1951), as shown by Table VII below.

Table VII.

Catechol amines in cat organs.

Organ	μg/g	
	Adrenaline	Noradrenaline
Heart	0.11; 0.25	0.80; 0.76
Spleen	0.17; 0.073	1.86; 1.6
Liver	0.050; 0.072	0.21; 0.16

Comment.

The use of aluminium oxide for adsorption has been found to be as effective as aluminium hydroxide under the conditions described. Elution with sulfuric acid offers the advantage of allowing biological assay of the extracts which is not the case when elution is made with acetic or oxalic acid.

The adsorption on aluminium oxide in alkaline medium (pH 8.5) probably depends on the formation of a coating of aluminium hydroxide on the surface of the aluminium oxide grains. During elution this layer is dissolved in acid together with the catechol compounds adsorbed, leaving the "carrier" grains behind, thus ensuring easy filtration. The amounts of aluminium salts in the eluate is insignificant for biological testing purposes.

Summary.

A method is described for the adsorption of catechol amines (adrenaline, noradrenaline) on aluminium oxide and elution with sulfuric acid.

The method involves only a few simple preparatory steps and gives an extract suitable for direct biological assay. The method has been successfully applied to urine and to organ extracts prepared with trichloroacetic acid.

The recovery of added catechol amines is about 80 % using biological assay. By subjecting the urine to acid hydrolysis for 20 minutes at 100°C in normal hydrochloric acid before adsorption, the total yield increased by 1—3 times in about 80 % of the samples.

The extracts obtained by elution with oxalic acid can be used for fluorimetric estimation of the catechol amines.

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Diurnal Variations in the Excretion of Free and Conjugated Noradrenaline and Adrenaline in Urine from Healthy Subjects.

By

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Quantitative information about the activity of the sympathetic adrenergic system and the adrenal medulla may be obtained by estimating the urinary output of adrenaline and noradrenaline. Previous investigations have shown that after administration of catechol amines they are excreted in a fairly constant proportion in the urine (EULER and LUFT, 1951; EULER, LUFT and SUNDIN, 1954). The excretion is largely independent of the diuresis. After bilateral adrenalectomy in man the adrenaline excretion is reduced by more than 80 per cent while no consistent changes occur in the noradrenaline excretion (EULER, FRANKSSON and HELLSTRÖM, 1954). This supports the conclusion that the urinary adrenaline figures give a relative measure of the suprarenal adrenaline secretion, while the noradrenaline output gives an indication of the activity of the adrenergic system. During insulin hypoglycemia only the adrenaline figures are increased (EULER and LUFT, 1952), while strenuous muscular work is accompanied by an increase in both adrenaline and noradrenaline excretion (EULER and HELLNER, 1951).

Diurnal variations in the excretion of noradrenaline and adrenaline may be expected to occur as a result of rhythmic variations in the activity of the adrenergic system and suprarenal medulla. It has previously been shown that during night rest the output

of both noradrenaline and adrenaline is considerably lower than during day hours (EULER and LUNDBERG, 1954). KÄRKI (1954) found the noradrenaline excretions to be 50—300 % higher during the day than during the nights, but noticed little difference in the adrenaline figures.

In the present paper the diurnal variations in healthy individuals in the age group 20—50 years are reported.

Material and Methods.

Urine was collected from 9 female and 6 male persons in good health. The output was measured during the period 23.00—08.00 at night, and then in three-hour periods during the rest of the day. In the female group 9 complete series of estimations were made comprising analysis of noradrenaline and adrenaline in untreated and hydrolyzed urine.

The urine was kept cold before extraction and the pH adjusted to 3—4. The catechol amines were adsorbed on aluminium oxide according to EULER and ORWÉN (1955), and eluted with sulfuric acid. The extracts were assayed on the cat's blood pressure and on the chicken rectal caecum against standards of noradrenaline and adrenaline and expressed as hydrochlorides in mμg (ng) per min. or μg per 24 hours (EULER and HELLNER, 1951; EULER, 1949). For extraction of free catechol amines the urine was untreated except for a brief heating to 100°C at pH 3—4 which often facilitated the further procedure without causing any splitting of conjugated catechol amines.

Total catechol amines (free and conjugated) were determined in urine after hydrolysis for 20 minutes at approximately pH 0 by adding 1/10 volume 10 N-H_2SO_4 and subjecting the urine to the same extraction procedure as for free catechol amines.

All excretion figures are uncorrected for the loss of about 20 % occurring in the extraction procedure.

I. Free Noradrenaline and Adrenaline in Urine.

The excretion of free catechol amines was determined in 9 individuals in the female group and in 5 individuals in the male group. The following diagram (Fig. 1) shows the variations during

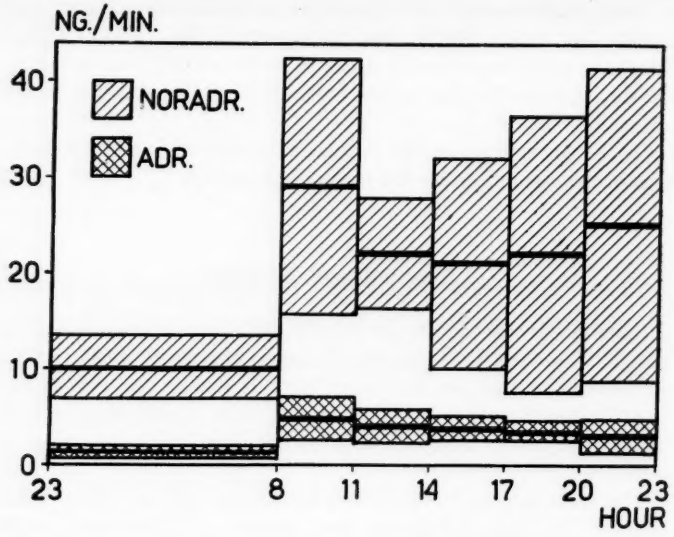

Fig. 1. Excretion of free adrenaline and noradrenaline in urine of 9 healthy female subjects. Mean figures ($\pm 3 \times$ S.E. of the mean). Ordinate: ng (mμg) per min. Abscissa: hours.

the different time periods for adrenaline and noradrenaline and the mean figures for each time period in the female group including three times the standard error of the mean in each direction.

As seen from the diagram noradrenaline constitutes by far the greater part of the excreted catechol amines at all times. In general the excretion during the night hours is less than half of that during the day hours. The figures obtained are similar to those previously reported for air force personnel during night and day hours (EULER and LUNDBERG, 1954).

The standard deviation is fairly high as might be expected. However, both for adrenaline and noradrenaline a statistically highly significant difference exists between the night and day figures, but no significant difference between the day periods.

No definite differences were noticed between males and females in the present material except possibly for a tendency to higher adrenaline figures in the afternoon in the males. (Fig. 2.)

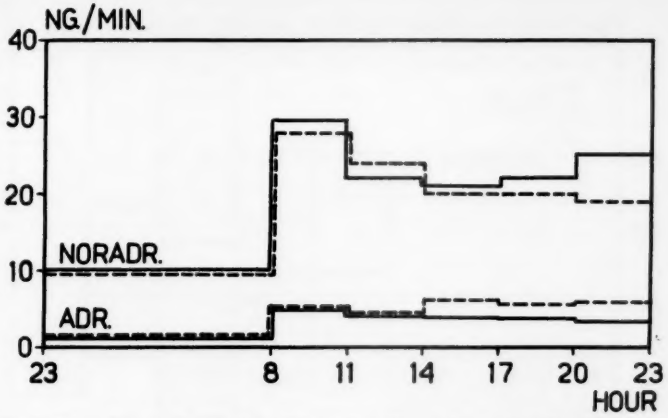

Fig. 2. Mean figures of excretion of free adrenaline and noradrenaline in urine of 9 female (full line) and 5 male subjects (broken line) in ng per min.

Table I.

Mean and range figures for free NA and A output in urine in normal individuals in ng/min.

	23—8	8—11	11—14	14—17	17—20	20—23
Female Adr. ...	1.2 (0.3—2.1)	4.8 (1.7—7.8)	4.0 (1.4—7.2)	3.8 (2.3—5.5)	3.5 (2.1—4.8)	3.1 (0.8—5.7)
Male ... Adr. ...	1.4 (0.2—4.0)	5.1 (3.2—7.6)	4.5 (0.8—6.7)	6.1 (1.9—14)	5.7 (1.9—8.1)	5.8 (1.2—8.1)
Female . Noradr..	10 (5.1—16)	29 (15—54)	22 (14—30)	21 (6.7—40)	22 (11—60)	25 (11—56)
Male ... Noradr..	9.2 (3—19)	28 (17—38)	24 (9—43)	24 (11—31)	20 (7.6—32)	19 (9.7—32)

II. Total Catechol Amines (Free and Conjugated).

The total catechol amines were estimated in 9 female and 6 male subjects.

Figure 3 shows the distribution of the noradrenaline and adrenaline figures in hydrolyzed urine. This is obviously quite similar to that of the free catechol amines though the figures are approximately twice as high.

The mean and range of all figures are given in Table II.

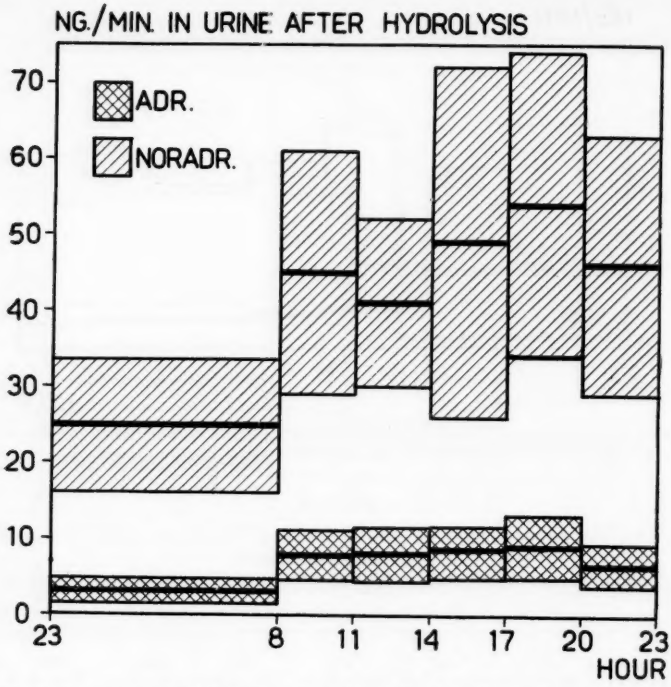

Fig. 3. Excretion of total (free and conjugated) adrenaline and noradrenaline in urine of 9 healthy female subjects. Mean figures ($\pm 3 \times$ S.E. of the mean). Ordinate: ng per min. Abscissa: hours.

Table II.

Mean and range of total (free and conjugated) NA and A output in urine in healthy individuals in ng/min.

Hours	23—8	8—11	11—14	14—17	17—20	20—23
Female . Adr.	3.1 (0.5—6.7)	7.8 (3.7—12)	8.0 (3.7—13)	8.3 (3.6—13)	9.0 (4.2—17)	6.5 (3.0—11)
Male ... Adr.	5.3 (1.9—8.7)	10 (6.0—21)	7.4 (1.3—13)	13 (4.7—20)	8.8 (3.9—14)	8.4 (2.0—14)
Female . Noradr..	25 (16—42)	45 (19—68)	41 (26—56)	49 (25—105)	54 (20—90)	46 (21—65)
Male ... Noradr..	42 (9.3—71)	49 (23—85)	57 (23—137)	55 (9—139)	44 (18—82)	54 (19—130)

There is a considerable spreading of the figures especially in the male group for noradrenaline probably connected with a wider range of variation in activity.

III. 24-Hour Excretion Figures.

The figures obtained for the 24 hour excretion in normal females and males are given in Table III, including the range.

Table III.

	Free		Total	
	Adr. µg/24 h.	Noradr. µg/24 h.	Adr. µg/24 h.	Noradr. µg/24 h.
Female ...	4.0 (1.7—5.6)	27 (15—50)	8.0 (3.6—11)	52 (30—71)
Male	5.0 (1.6—8.0)	26 (15—38)	11 (4.1—17)	69 (22—129)

The excretion figures for free catechol amines per 24 hours do not show any differences between the sexes. When compared with other similar series the noradrenaline figures show good agreement (EULER and HELLNER, 1951; KÄRKI, 1954), though the adrenaline figures are lower. The higher figures found by KÄRKI may have been due chiefly to a systematic error in the assay on the chicken rectal caecum which give too high estimates, if pH of the standard adrenaline solution is below pH 4.0 on account of an inhibition of the response as noted by HÖKFELT in our laboratory.

Summary.

The average urinary excretion of free noradrenaline in a material of 14 healthy young individuals was 27 µg/24 hours (range 15—50) and of adrenaline 4.3 µg/24 hours (range 1.6—8.0).

The average adrenaline excretion during the night hours (23.00—08.00) was 1.2 mµg/min. (range 0.2—4.0) as against 4.6 (range 0.8—14) during the day hours ($p < 0.001$).

Similarly the noradrenaline excretion was significantly lower during the night hours (9.8 mµg/min., range 3—19) than during the day hours (23 mµg/min., range 7.6—60).

Total catechol amines (free and conjugated) were estimated after subjecting the urine to hydrolysis in 1 N sulfuric acid for 20 min. The figures showed approximately the same distribution as the free catechol amines but were about twice as high.

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Effect of Increased Adrenergic Nerve Activity on the Content of Noradrenaline and Adrenaline in Cat Organs.

By

U. S. VON EULER and S. HELLNER-BJÖRKMAN.

Several facts indicate that the resynthesis of the adrenergic neurotransmitter occurs rapidly after its release, though little is known about the details. LUCO and GONI (1948) showed that the content of sympathomimetic substances in the splanchnic nerves was largely unchanged after prolonged electrical stimulation.

Since the release and synthesis of the adrenergic neurotransmitter are of greater physiological significance at the nerve terminals than in the nerve trunk, we have undertaken some experiments in order to study the effect of increased adrenergic nerve activity on the noradrenaline content of the spleen, heart and liver of the cat. Since the nerve transmitter will then be released in considerable quantities as borne out by the classical experiments of CANNON and ROSENBLUETH, the noradrenaline content of the organ will give an indication as to whether or not the resynthesis process is capable of keeping the stores at the normal level. The organ content may be used for the purpose of evaluating the amount in the nerve terminals, since it has been shown that the organ loses its catechol amines after degeneration of the adrenergic nerves (CANNON and LISSÁK, 1939; GOODALL, 1951; EULER and PURKHOLD, 1951) and replenishes its content upon regeneration of these nerves.

Methods.

All experiments have been made on cats, anaesthetized with nembutal. The increase in adrenergic nerve activity has been brought about by 1) electrical stimulation of a branch of the splenic nerves 2) prolonged carotid artery occlusion or denervation of the carotid sinuses. The organs were prepared and the extracts tested according to the method described previously (EULER, 1949).

Results.

1. *Electrical stimulation of splenic nerves.*

The main branch of the nerves to the caudal portion of the spleen was dissected free for about 1 cm and tied centrally. The stimulating square wave current had a frequency of 40 c/s, a pulse duration of 5 msec. and a voltage adjusted to give a nearly maximal contraction of the stimulated part of the spleen. The stimulus was applied by platinum electrodes for 10—20 minutes. After this time the contracted and the uncontracted parts of the spleen were separated and prepared. In one case stimulation was applied to the sympathetic in the neck on one side and the two submaxillary glands analysed for their catechol amine content.

The results are shown in Table I.

Table I.

Noradrenaline and adrenaline content of stimulated and unstimulated portions of spleen and submaxillary gland in the cat.

Organ	Stim. time min.	Stimulated		Unstimulated		Noradr. Ratio stimulated unstimulated
		Adr. µg/g	Noradr. µg/g	Adr. µg/g	Noradr. µg/g	
Spleen	10	0.005	1.3	—	—	—
»	10	0.020	1.2	0.050	0.77	1.6
»	20	0.044	0.18	0.020	0.39	0.46
»	15	0	1.6	0.092	0.76	2.1
»	10	0.046	1.7	0.020	1.7	1.0
Submax. gland.	10	0.040	1.4	0.11	1.4	1.0

In two instances there was no change, while the content was higher after stimulation in two cases and lower in one. In the latter case the condition of the animal was bad owing to a large

hemorrhage accompanied by a fall in blood pressure. This case showed absolute figures which were considerably lower than the control figures.

The experiments gave no indication that stimulation and increased release of the neurotransmitter cause a fall in the catechol amine content of the organ. In the two cases in which an increase was observed the stimulated portion was very firmly contracted, which reduced the mass in comparison with that of the unstimulated portion.

2. *Carotid artery occlusion and carotid sinus denervation.*

Of the six experiments performed the blood pressure was well maintained in five. The results are shown in Table II.

Table II.

Noradrenaline and adrenaline in cat organs after carotid artery occlusion and carotid sinus denervation.

No. of experiments	Spleen		Heart		Liver		Remarks
	Adr. µg/g	Noradr. µg/g	Adr. µg/g	Noradr. µg/g	Adr. µg/g	Noradr. µg/g	
1	0	1.5	0.016	1.2	0.006	0.36	Both carotid arteries clamped for 45 min.
2	0.020	1.5	0.014	0.65	0.0034	0.014	»
3	0	1.0	0.026	1.0	0.0046	0.066	Denervation of both carotid sinuses, vagotomy 1 hour
4	0	2.5	0.010	0.8	0.015	0.019	ib., 4 hours
5	0.030	1.3	0.11	1.6	0.053	0.39	»
Mean	0.010	1.6	0.035	1.1	0.016	0.17	
Control figures (EULER & HELLNER-BJÖRKMAN, 1955).....	0.027	1.2	0.046	0.70	0.009	0.24	
(EULER & UDDÉN, 1951)		1.6		0.62		0.12	

From Table II it can be seen that there is no indication of a fall in the catechol amine content in the spleen and the heart in spite of an increased release. On the contrary the figures tend to increase in the heart.

Comment.

The experiments described are in agreement with the well-known fact that adrenergic nerve activity can be maintained over long periods without loss in efficiency. The persistent hypertension in dogs deprived of their buffer nerves bears out this fact clearly. Our experiments have shown that prolonged overactivity of the adrenergic nerves does not normally lead to a fall in their noradrenaline as reflected by the content of the organ. The result allows the conclusion that the transmitter is rapidly and continuously synthesized when the release is enhanced.

In one experiment in which the blood pressure was not maintained after carotid sinus denervation the catechol amine figures were very low in the spleen and the heart (less than half of normal) while they were normal or even increased in the liver.

No consistent alterations of the catechol amine content of the heart and spleen were observed during one hour periods of hypoxia, induced by letting the cats breathe a mixture of 10 per cent oxygen in nitrogen.

Summary.

Electric stimulation of the splenic nerves in the cat caused no consistent changes in the noradrenaline and adrenaline content of the stimulated portion of the spleen in comparison to the non-stimulated portion.

Increased reflex adrenergic activity elicited by carotid artery occlusion or carotid sinus denervation caused no significant change in the noradrenaline content of the spleen, heart and liver of the cat.

It is concluded that release of the adrenergic transmitter is normally followed by rapid resynthesis, allowing the stores to be maintained at an approximately constant level.

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Effect of Amine Oxidase Inhibitors on the Noradrenaline and Adrenaline Content of Cat Organs.

By

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It has been shown that most mammalian organs contain noradrenaline and adrenaline in amounts which vary greatly in different organs but are relatively constant for the same organ (see EULER, 1951, HÖKFELT, 1951, and others). By comparing the noradrenaline content in an organ and its main nerve branches it can be concluded that the noradrenaline is derived from the terminal parts of the adrenergic nerves, but even the nerve trunks to the organ contain considerable amounts (EULER, 1951). After postganglionic sympathetic denervation the noradrenaline disappears from the organs or falls to very low figures (GOODALL, 1951; EULER and PURKHOLD, 1951). The origin of the adrenaline in organs is still under dispute; however, it diminishes less than noradrenaline after denervation of an organ, which suggests that it is derived from extraneural sources, presumably chromaffin cells.

The opinion has been expressed that the nerve endings possess some specific structures containing noradrenaline in a high concentration (EULER, 1954). The demonstration of specific secretion granules in the chromaffin cells (HILLARP, LAGERSTEDT and NILSON, 1953, BLASCHKO and WELCH, 1953) lends support to the view that similar structures may be present in the nerve terminals. This is also supported by the fact that the postganglionic neurons represent homologues of the chromaffin cells and that both release catechol amines upon stimulation.

The possible rôle of amine oxidase as the normal inactivator of the released transmitter has recently been suggested and emphasized by BURN and ROBINSON (1951) and BURN (1952). Although there is not complete agreement as to the rôle of amine oxidase in this respect, sufficient data as to its occurrence and action (THOMPSON and TICKNER, 1951; BLASCHKO, 1952; KOELLE and VALK 1954) are available to warrant the view that it may be connected with the normally occurring inactivation of the transmitter at or near the site of action.

The present work was undertaken in order to obtain some information as to whether or not amine oxidase inhibitors influence the catechol amine content of an organ.

Material and Methods.

The catechol amine content was determined in the spleen, the heart and the liver of the cat, since these organs have been found to contain a fairly constant amount of noradrenaline and adrenaline though at different levels. The cats were anaesthetized with 0.35 g nembutal per kg intraperitoneally and the organs taken out in the controls after 1—2 hours. In other animals various amine oxidase inhibitors (propamidine, methamphetamine, ephedrine, cocaine, choline-p-tolyl-ether)[1] (BLASCHKO, 1954) were injected in varying doses 20—60 min. before removing the organs. These were freed from blood by gentle pressure and were then weighed. The organs were minced in a Waring blendor with 2—10 p.c. trichloracetic acid. After filtration the catechol amines were adsorbed on aluminium hydroxide (EULER, 1949) or aluminium oxide (EULER and ORWÉN, 1955) and eluted. The extracts were biologically tested for adrenaline and noradrenaline differentially by using the cat's blood pressure and the chicken rectal caecum. The adrenaline and noradrenaline figures are given in terms of the hydrochlorides in μg per g organ and in μg per kg body weight.

Results.

The adrenaline and noradrenaline figures in each organ in the controls and after administration of 6—30 mg propamidine isethionate (kindly placed at our disposal by May & Baker Ltd.) and 5—20 mg methamphetamine sulfate are given in Table I.

[1] Our thanks are due to Dr. P. HEY, Pharmacol. department, Univ. of Leeds for a generous supply of choline-p-tolyl-ether.

Table I.

Adrenaline and Noradrenaline in μg/g organ and in μg/kg body weight in cat organs after propamidine (6—30 mg/kg), methamphetamine (5—20 mg/kg) and in controls.

	Adrenaline μg/g organ	Noradr. μg/g organ	% Adr.	Adrenaline μg/kg body wt.	Noradr. μg/kg body wt.	Organ
Propamidine	0.020 (0—0.033) n = 7	1.3 (0.5—2.8)	1.7 (0—3.5)	0.036 (0—0.080)	2.7 (1.0—5.2)	Spleen
Methamphetamine	0.025 (0—0.052) n = 6	1.7 (0.82—2.5)	1.8 (0—6.0)	0.052 (0—0.11)	3.9 (1.5—6.4)	»
Controls	0.027 (0—0.073) n = 8	1.2 (0.75—2.1)	1.9 (0—5.3)	0.051 (0—0.14)	2.9 (1.1—5.1)	»
» Euler & Uddén, 1951		1.6 (1.3—1.7) n = 5			3.0 (2.2—3.5)	»
Propamidine	0.024 (0.002—0.051) n = 6	1.2 (0.59—2.0)	2.8 (0.1—7.0)	0.098 (0.01—0.23)	4.9 (2.3—8.4)	Heart
Methamphetamine	0.020 (0.013—0.025) n = 4	0.82 (0.64—0.99)	2.5 (1.4—3.3)	0.13 (0.054—0.24)	4.7 (2.8—7.2)	»
Controls	0.046 (0.015—0.083) n = 4	0.70 (0.58—0.85)	6.2 2.2—8.8)	0.18 (0.07—0.32)	2.8 (2.1—3.3)	»
» (Euler & Uddén, 1951)		0.62 (0.5—0.8) n = 5			2.6 (1.8—4.5)	»
Propamidine	0.018 (0.002—0.037) n = 7	0.39 (0.094—0.66)	5.2 (0.4—9.8)	0.53 (0.052—1.5)	11 (2.6—26)	Liver
Methamphetamine	0.012 (0.003—0.027) n = 6	0.19 (0.09—0.38)	9.0 (0.9—18)	0.20 (0.063—0.32)	4.9 (1.8—7.5)	»
Controls	0.009 (0—0.030) n = 9	0.24 (0.028—0.40) n = 11	3.9 (0—9.3)	0.21 (0—0.57)	6.3 (0.7—9.3)	»
» (Euler & Uddén, 1951)		0.12 (0.03—0.2)			2.4 (0.7—3.7)	»

The table also includes some control figures from previous work (EULER and UDDÉN, 1951) for comparison.

In the spleen no significant differences were noted as a result of injection of propamidine or methamphetamine.

In the heart, however, four out of six noradrenaline figures of the propamidine series were in excess of the highest figure noted in the controls. A similar tendency was also observed for the liver. With the different doses which were used no differences in the effect on the catechol amine content were noticed.

Some experiments were also made with other amine oxidase inhibitors (cocaine, ephedrine, choline-p-tolyl-ether), but no significant alterations in the catechol amine content of the organs were observed.

The effect of increased adrenergic activity on the catechol amine content of the organs in the presence of amine oxidase inhibitors was also investigated. An increased release of the adrenergic transmitter was elicited by direct electric stimulation of a branch of the splenic nerves and by carotid occlusion or carotid denervation.

In none of these experiments was any significant difference in the catechol amine content noted in the stimulated and unstimulated part of the spleen or in the organs compared with the controls after injection of 10 and 20 mg/kg of propamidine.

Comment.

Since no significant differences in the content of adrenaline and noradrenaline were observed in cat organs as a result of injections of even large quantities of amine oxidase inhibitors, with the possible exception of propamidine on the heart, it is concluded that the process of formation and the storage function of these catechol amines is not affected to any great extent by amine oxidase, which may act only after the release of the neurotransmitter.

This conclusion is further supported by the experiments in which the catechol amine content was measured after increased activity in the presence of propamidine.

Summary.

The effect of various amine oxidase inhibitors (propamidine, methamphetamine, choline-p-tolyl-ether, ephedrine, cocaine) on

the content of noradrenaline and adrenaline of the heart, liver and spleen of the cat has been studied.

In no instance were highly significant changes observed, though increased noradrenaline figures in the heart were observed after propamidine in several cases. Increased adrenergic nerve activity caused by carotid occlusion, carotid sinus denervation or electrical stimulation of the splenic nerves had no consistent effect on the catechol amine content of the organs in the presence of amine oxidase inhibitors.

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The Rôle of Amine Oxidase in the Inactivation of Catechol Amines Injected in Man.

By

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RICHTER (1940) and RICHTER and MACINTOSH (1941) showed that after administration of catechol amines in man and animals a certain proportion is excreted in the urine, partly in the free and partly in the conjugated form. Quantitative estimations of the urinary output during and after intravenous continuous infusion of adrenaline and noradrenaline in man have since been made by EULER and LUFT (1951), GOLDENBERG (1951), and EULER, LUFT and SUNDIN (1954). The amounts excreted in the urine were in the range of 1—4 p.c. of the quantity administered during the same time. After subcutaneous and intramuscular injection of adrenaline and noradrenaline in man the proportion excreted in the urine was similar to that excreted after intravenous infusion (LILJEDAHL, 1955), the period of excretion being extended over three hours. The low excretion figures indicate that the greater part of these catechol amines injected are inactivated in the body. If variations occur in the percentage appearing in the urine after administration of various catechol amines this might then give some information as to the inactivation rate.

This appears to be particularly interesting from the point of view of the possible inactivation paths for catechol amines in the body. It has been postulated that amine oxidase is the main effective inactivating enzyme for catechol amines. Support for this concept has been presented by BURN and ROBINSON (1957, 1952) and BURN (1952). The possibility of several systems

working under different conditions should be kept in mind, however. Thus the inactivation of noradrenaline released from the adrenergic nerve endings may proceed by means of a different mechanism than that operating in the case of circulating adrenaline or noradrenaline, administered either exogenously or endogenously from the suprarenals.

A way of studying the possible rôle of amine oxidase in the breakdown of circulating catechol amines would be to compare the relative excretion rate of adrenaline or noradrenaline on the one hand and dihydroxynorephedrine (Cobefrine, Corbasil) on the other, since the latter compound is not attacked by amine oxidase.

Methods.

The injections were made on five healthy male and one female medical student (No. IV). Urine was collected for a period of 3 hours before injections in order to obtain the basal excretion, and during the two hours following the injection.

The catechol amines used were l-adrenaline hydrochloride (Exadrin, Astra), dl-noradrenaline hydrochloride (Norexadrin, Astra) and l-dihydroxynorephedrine hydrochloride (Corbasil, Bayer), which were kindly supplied by A.B. Astra and Bayer Co. Of all substances 0.5 mg was injected subcutaneously at the end of the control period. The free catechol amines were prepared by adsorption on aluminium oxide according to EULER and ORWÉN (1955) and estimated biologically according to EULER (1949). Corbasil was estimated by utilizing the activity ratio corbasil : noradrenaline found in each experiment and subtraction of the adrenaline and noradrenaline figures during the control period. The assumption that the extra output is due to Corbasil is supported by results obtained by paper chromatography and by comparison of the fluorimetric and biologic estimation of the catechol amines in the urine extracts. All figures are given in terms of the hydrochlorides of the levo-isomers.

1. *Adrenaline.*

Six experiments were made in which 0.5 mg l-adrenaline hydrochloride were injected subcutaneously. Table I shows the excretion figures.

Table I.

Excretion of adrenaline and noradrenaline in urine after subcutaneous injection of 0.5 mg adrenaline.

Subject	Hours	Excretion μg		Extra output μg		Per cent of injected adr. excreted in two hours
		Adr.	Noradr.	Adr.	Noradr.	
I	Control	1.8	1.9			
	1st hour	8.1	4.3	6.3	2.4	2.0 %
	2nd »	3.5	2.7	1.7	0.8	
	after inj.					
II	Control	0.39	1.3			
	1st hour	7.2	2.5	6.8	1.2	3.4 %
	2nd »	11.4	5.7	11.0	4.4	
	after inj.					
III	Control	0.40	1.3			
	1st hour	7.1	3.9	6.7	2.6	1.8 %
	2nd »	2.8	2.9	2.4	1.6	
	after inj.					
IV	Control	0.38	2.6			
	2 hours a. i.	12.4	2.5	12	— 0.1	2.4 %
V	Control	0.18	4.4			
	2 hours a. i.	10.8	0	10.6	— 4.4	2.1 %
VI	Control	0.44	0.8			
	2 hours a. i.	11.4	0	11	— 0.8	2.2 %

m = 2.3 %

2. *Noradrenaline.*

In six experiments 0.5 mg dl-noradrenaline hydrochloride were injected subcutaneously (0.25 mg of the l-isomer). The results are given in Table II.

3. *Corbasil.*

Five experiments were made with Corbasil, 0.5 mg of the levo-isomer being injected subcutaneously. The results are shown in Table III.

Comment.

The excess output of catechol amines in urine after injection of adrenaline and noradrenaline is of the same order as that previously observed. The adrenaline figures are slightly higher

Table II.

Excretion of adrenaline and noradrenaline in urine after subcutaneous injection of 0.25 mg noradrenaline.

Subject	Hours	Excretion μg		Extra output μg		Per cent of injected noradr. excreted in two hours
		Adr.	Noradr.	Adr.	Noradr.	
I	Control	0.39	3.8			
	1st hour	1.4	13	1.0	9.5	3.8 %
	2nd »	1.9	3.9	1.5	0.1	
	after inj.					
II	Control	0.42	1.7			
	1st hour	1.5	6.1	1.1	4.4	2.3 %
	2nd »	0	3.1	0	1.4	
	after inj.					
III	Control	0.52	2.5			
	1st hour	2.5	20	2.0	17.5	7.7 %
	2nd »	1.8	4.2	1.3	1.7	
	after inj.					
IV	Control	0.16	3.0			
	2 hours a. i.	0.94	10.4	0.78	7.4	2.9 %
V	Control	0.30	1.9			
	2 hours a. i.	0.63	11.1	0.33	9.2	3.7 %
VI	Control	0.24	3.0			
	2 hours a. i.	0.71	9.8	0.46	6.8	2.7 %

m = 3.8 %

than those obtained when adrenaline was administered by continuous intravenous injection (Euler, Luft and Sundin, 1954).

For Corbasil the output figures were of the same order as those observed for adrenaline and noradrenaline. On the assumption that Corbasil is not attacked by amine oxidase, it follows that the rôle of this enzyme for the inactivation of circulating catechol amines in general in the body is rather insignificant, since about 95 p.c. of the administered Corbasil is inactivated in a different way. Conjugation of Corbasil is probably not an important pathway of elimination, since hydrolysis did not increase the yield more than about 30 p.c. which is approximately the same increase as for adrenaline and noradrenaline.

The experiments described in the present report were not designed to give information about the rôle of amine oxidase for the inactivation of topically released noradrenaline. They

Table III.

Excretion of Corbasil in urine after subcutaneous injection of 0.5 mg Corbasil.

Subject	Hours	Excretion μg		Extra output μg		Per cent of injected Corbasil excreted in two hours
		Adr.	Noradr. equiv.	Adr.	Corbasil calc.	
I	Control	0.60	0.90			
	1st hour	2.6	13	2.0	16	
	2nd »	0.70	3.4	0.10	3.3	3.9 %
	after inj.					
II	Control	0.46	1.2			
	1st hour	0.87	14	0.4	17	
	2nd »	1.0	3.9	0.6	3.6	4.1 %
	after inj.					
IV	Control	0.26	1.3			
	2 hours a. i.	2.7	12	2.4	16	3.2 %
V	Control	0.36	3.4			
	2 hours a. i.	2.8	11	2.4	14.5	2.9 %
VI	Control	0.31	1.9			
	2 hours a. i.	2.4	13	2.1	18.5	3.7 %

m = 3.6 %

suggest, however, that circulating catechol amines are probably inactivated only to a minor degree by amine oxidase and that some other inactivating system must therefore be considered. The rôle of peroxidases in this connection has not been elucidated but should be considered on account of their ubiquity and ability to transform catechol amines into inactive compounds.

This is also in keeping with the relatively unimportant effect of amine oxidase inhibitors on the action of injected catechol amines such as adrenaline and noradrenaline.

Summary.

The urinary excretion of adrenaline, noradrenaline, and dihydroxynorephedrine (Corbasil, Cobefrine) after subcutaneous injection in man was studied.

Of the adrenaline 2.3 per cent, of the noradrenaline 3.8 per cent, and of Corbasil 3.6 per cent, on an average, of the injected amount

was excreted in free form in urine during the two hour period following the injection.

There was no indication of any appreciable quantities being excreted in conjugated form.

Two hours after the injection, the urinary excretion of the catechol amines had returned to the basic level.

It is concluded that, since injected Corbasil is inactivated to about the same extent as noradrenaline or adrenaline in the organism although it is not attacked by amine oxidase, some other enzyme system plays a significant rôle in the destruction of circulating catechol amines.

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Biologically Inactive Catechol Derivatives In Urine.

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During attempts to apply the ethylene diamine condensation method of NATELSON, LUGOVOY and PINCUS (1949) to fluorimetric estimation of catechol amines (adrenaline and noradrenaline) in urine it was noted that the total figures expressed as noradrenaline were up to 100 times higher than those obtained by biological assay. Experiments were therefore made in order to obtain information about these products.

I. *Fluorimetric estimations in urine extracts.*

Extracts of urine samples were made by adsorption on aluminium oxide according to the principle introduced by SHAW (1938), and using the procedure described by EULER and ORWÉN (1955). The urine (100 ml) was briefly heated to 100°C at pH 3, and the dihydroxyphenols adsorbed on 2.5 g aluminium oxide (Brockmann, for chromatography) by adding sodium hydroxide to pH 8.5. The adsorbed dihydroxyphenols were eluted with 2×5 ml 0.25 N sulfuric acid, and the fluorescence was measured after condensation with ethylene diamine according to the procedure used by WEIL-MALHERBE and BONE (1952).

Aluminium oxide adsorbs dihydroxyphenyl compounds with a high degree of specificity, and since the fluorescence figures found in urine extracts were far in excess of those which could be expected from the noradrenaline present, it was suspected that either dopamine (which is 50—100 times less active than noradrenaline

on the cat's blood pressure) or some degradation product of dopa or catechol amines caused the high fluorescence figures.

Dopamine has been shown to occur in urine (HOLTZ, CREDNER and KOEPP, 1942; HOLTZ, CREDNER and KRONEBERG, 1947; EULER, HAMBERG and HELLNER, 1951). Deamination products of catechol amines might also occur as a result of the action of amine oxidase. 2 : 5-dihydroxyphenyl-acetic (homogentisic) acid was used in some experiments and later 3 : 4-dihydroxyphenyl-acetic acid (kindly placed at our disposal by Dr. R. Dahlbom, Astra Ltd., Södertälje) for comparison with the urine catechols.

Table I gives the figures obtained by fluorimetric estimations of urine extracts, expressed in terms of noradrenaline, dopamine and catechol acetic acid compared with the noradrenaline figures found by bioassay. The biological assay figures in hydrolyzed samples have been doubled owing to racemization.

Table I.

Comparison of biological estimation of noradrenaline and fluorescence equivalents of noradrenaline, dopamine and 3 : 4, dihydroxyphenyl acetic acid in unselected extracts of urine.

Biological assay of noradrenaline mg/24 hours	Fluorescence equivalents of			
	Noradrenaline mg/24 hours	Dopamine mg/24 hours	3 : 4, dihydroxyphenyl acetic acid mg/24 hours	
0.039	1.4	4.3	0.84	Nonhydrolyzed
0.016	1.1	3.4	0.66	»
0.022	0.33	0.99	0.20	»
0.014	0.92	2.8	0.55	»
0.025	0.74	2.2	0.44	»
0.027	0.85	2.6	0.51	»
0.030	0.45	1.4	0.27	»
0.041	1.7	5.2	1.0	»
0.016	0.67	2.0	0.40	»
0.017	2.2	6.5	1.3	»
0.046	8.7	26	5.2	Hydrolyzed (20 min. 100°C N-H_2SO_4)
0.060	13	38	7.8	»
0.036	2.8	8.5	1.7	»
0.042	9.6	29	5.8	»
0.050	5.4	16	3.2	»
0.068	8.6	26	5.2	»
0.120	5.4	16	3.2	»
0.082	13	38	7.9	»
—	6.2	19	3.7	»
—	9.5	28	5.7	»

As seen in the Table the fluorescence figures expressed in noradrenaline equivalents are 10—100 times higher than those found by biological assay. In hydrolysed urine samples (20 min. 100°C, N-sulfuric acid) the ratio was even higher. The dopamine equivalents were in better agreement with the biological assay figures provided that all activity was due to dopamine, which, however, has been excluded by chromatographic and other tests. The quantitative relationship between the figures expressed in dihydroxyphenyl acetic acid and the biologically estimated noradrenaline on the other hand is of the same order as that found chromatographically in urine extracts.

II. *Paper chromatography of urine extracts.*

Extracts of 100 ml urine adsorbed on aluminium oxide were taken up in acid ethanol and subjected to paper chromatography using ascending runs with *n*-butanol/1N-hydrochloric acid as solvent. After drying, the paper was treated with ethylene diamine which transformed the dihydroxyphenyl compounds into the fluorescent compounds. Of the fluorescent spots noradrenaline and dopamine were identified by their R_F-values. A large, strongly fluorescent spot was observed near the solvent front. In other runs this was compared with homogentisic and catechol acetic acid which gave similar spots at approximately the same place. The general chromatographic behaviour of the substance giving the fast-moving fluorescent spot in urine extract chromatograms suggests that it is closely related or identical with catechol acetic acid, which is formed by oxidative deamination of dopamine and subsequent oxidation by aldehyde oxidases in the tissues (Holtz and Kroneberg, 1950). A compound showing similar properties was formed in large amounts during incubation of dopa with liver tissue, as indicated by paper chromatograms.

III. *Column chromatography of urine extracts.*

The catechol compounds in 2—5 litres of urine were adsorbed on aluminium oxide, eluted, concentrated and taken up in *n*-butanol. The extract was subjected to column chromatography on starch according to Moore and Stein (1942) with *n*-butanol-0.1 N-HCl-acetic acid as solvent. 300 ml 10 N acetic acid and ascorbic acid to a concentration of 0.1 % were added to 900 ml of the *n*-butanol saturated with 0.1 N-HCl. Figure 1 shows the fluorescence values of a series of 2.5 ml fractions. The strongest fluo-

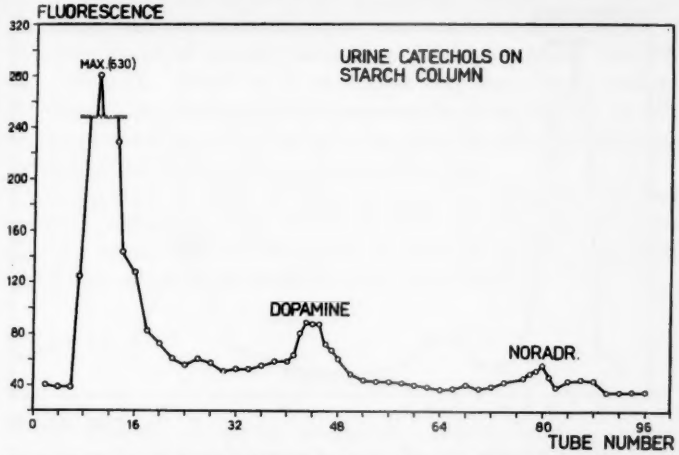

Fig. 1. Chromatography of urine catechols on starch column. Solvent *n*-butanol-0.1 N-HCl-acetic acid. Dopamine and noradrenaline identified biologically and by position. Fluorescence peak in tube 10 identified as catechol acetic acid.

rescence occurred at $R = 0.78$, which was also the case with 3 : 4-dihydroxyphenyl acetic acid and the product formed by incubation of guinea-pig's liver with dopa.

Fluorescence peaks were also obtained at positions indicating dopamine and noradrenaline which have previously been demonstrated in urine extracts by EULER, HAMBERG and HELLNER (1951) with a similar technique.

IV. *Chromatography of degradation products of dopamine.*

Dopa 40 μg per ml and 5 g sliced liver tissue from guinea-pig were incubated for two hours at 37°C in 20 ml Tyrode solution. After the end of the incubation the contents were extracted with $1^1/_2$ volume of 5 % trichloracetic acid and the catechol compounds adsorbed on aluminium oxide and eluted. The extract was evaporated *in vacuo*, taken up in acid ethanol and placed on paper for chromatography using the same solvent system as above.

After treatment with ethylene diamine the fluorescent spots caused by dopa and dopamine were identified by their positions. In addition, a large fluorescent spot near the solvent front was found, having the same position as that given by urine extracts and by catechol acetic acid. Liver incubated without dopa did not give this spot. It is concluded that the large fluorescent spot

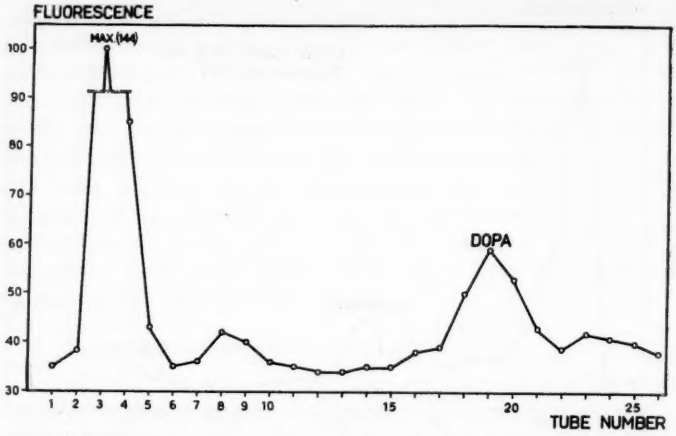

Fig. 2. Chromatography on starch column of extract of guinea-pig's liver incubated with dopa for 2 hours. Solvent *n*-butanol-0.1 N-HCl-acetic acid. Ordinate: Fluorescence units, Abscissa: number of tubes.

is due to 3 : 4-dihydroxyphenyl acetic acid. The aldehyde could be excluded since this compound causes a fall in blood pressure in test animals as found by HOLTZ, CREDNER and KOEPP (1942).

Column chromatography on starch with extracts of guinea-pig's liver incubated with dopa gave the typical fluorescence peak of catechol acetic acid (Fig. 2).

Comment.

Normal urine contains relatively large amounts of dopamine, probably formed from dopa by decarboxylation. A certain proportion of the dopamine in the organism would be expected to be deaminated by amine oxidase and subsequently transformed into catechol acetic acid and excreted in the urine. The chromatographic behaviour of the catechol derivative causing the larger part of the fluorescence of urine extracts after treatment with ethylene diamine suggests that it is catechol acetic acid.

Of other compounds catechol acetaldehyde and catechol glycollic acid or the corresponding aldehyde might be considered. The aldehydes lower the blood pressure and since an effect of

this kind was not noticed in the urine extracts the quanti y of this compound, if present, must have been quite small. Catechol glycollic acid, ormed in an analogous way from noradrenaline, migrates in chromatographic experiments in a way similar to that of catechol acetic acid. The catechol glycollic acid was obtained by incubation of liver tissue with noradrenaline.

The presence in urine of large amounts of biologically inactive catechol compounds make the fluorescence reaction with ethylene diamine unsuitable for estimation of catechol amines, a method which has been suggested by MANGER et al. (1953).

Summary.

The normally occurring biologically active catechol amines (dopamine, noradrenaline and adrenaline) form only a small part of the total catechol compounds in urine as evidenced by a comparison of the biological activity and the strength of the fluorescence reaction after condensation with ethylene diamine according to NATELSON et al.

While acid hydrolysis of urine increased the biologically assayed noradrenaline 2—3 times the fluorescence figures were increased about 8 times, suggesting that a large proportion of the inactive catechols is conjugated.

The catechol-acetic acid formed by incubation of guinea-pig liver tissue with dopamine agrees chromatographically with the catechol compound occurring in urine which is responsible for the greatest part of the fluorescence after condensation with ethylene diamine.

The ethylene diamine fluorescence reaction is not suitable for estimations of adrenaline and noradrenaline in urine.

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Inactivation of Catechol Amines by Liver Tissue in Vitro.

By

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Fluorimetric estimation of catechol compounds in normal urine after condensation with ethylene diamine according to NATELSON et al. (1949) has shown that they consist to the largest part of the end product of oxidative deamination of dopamine, catechol acetic acid (EULER, EULER and FLODING, 1955). The biologically active amines, noradrenaline, adrenaline and dopamine only form a small part of the total of the fluorescent products.

By using chromatography on starch column according to MOORE and STEIN (1952) it has been possible to separate and partly identify the breakdown products of some catechol amines and dopa after incubation with guinea-pig liver tissue, which is known to contain amine oxidase (BLASCHKO, RICHTER and SCHLOSSMAN, 1937; BAIN, GAUNT and SUFFOLK, 1937; HOLTZ and KRONEBERG, 1950, and BLASCHKO, 1952).

Methods.

l-dopa, l-noradrenaline bitartrate (kindly supplied by A.G. Sanabo, Wien) and Corbasil (Bayer) (l-3 : 4, dihydroxynorephedrine) hydrochloride were used in the incubation experiments. 5 g sliced guinea-pig liver was immersed in 20 ml Tyrode solution to which was added 4 mg of the above mentioned compounds. Incubation was performed at 37°C for 2 hours. At the end of this period $1^1/_2$ volumes of 5 per cent trichloracetic acid was added

and the mixture filtered. The filtrate was then treated with 2.5 g aluminium oxide at pH 8.5 for adsorption of the catechol compounds according to EULER and ORWÉN (1955). The catechols were eluted with 2 × 5 ml 0.25 N-H_2SO_4 and the eluate evaporated in a vacuum exsiccator to a small volume. This was extracted with 3—5 ml methanol, filtered, and 2—4 ml *n*-butanol added to the filtrate. The solution was evaporated until the methanol was removed.

As solvent for the chromatography on the starch column was used *n*-butanol saturated with 0.1 N-HCl, to which was added $^1/_3$ volume 10-N acetic acid and 1 g ascorbic acid per l. Chromatography was carried out with an automatic fraction collector in equal portions of 100—300 drops at a speed of 5—8 drops per minute.

Of the samples 0.05—0.2 ml were treated according to the procedure of WEIL-MALHERBE and BONE (1952) for condensation of the catechol compounds with ethylene diamine and the relative fluorescence read against a blank and quinine sulfate standard in 0.1 N-H_2SO_4.

For biological assay the samples were evaporated to a small volume in vacuo at low temperature after addition of water. The residue was dissolved in 2 ml Tyrode solution, and tested on the cat's blood pressure. Paper chromatograms were made with *n*-butanol/1 N HCl as solvent, and run for 20—25 hours (ascending).

Experimental.

1. *Dopa.* The results of chromatographic separation on starch column of the extract of liver incubated with l-dopa are illustrated in Figure 1, which gives the fluorescence after condensation with ethylene diamine. Three experiments included in figure 1 show the peaks of dopa, dopamine and catechol acetic acid at tube numbers 18—20, 13—15, and 3—4 respectively. The different products were identified by paper chromatography and biological assay. The peak at the tubes 7—8 was presumably catechol acetaldehyde since this compound occurs as the first step of the oxidative deamination of dopamine and is characterized by a lowering effect on the blood pressure (HOLTZ, CREDNER and KOEPP, 1942).

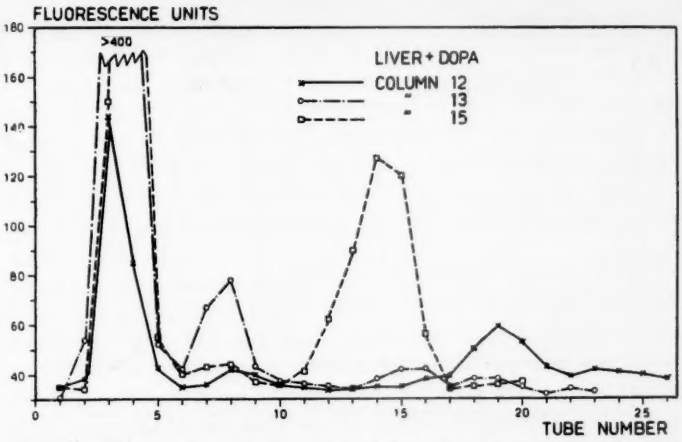

Fig. 1. Chromatograms on starch column of three extracts of guinea-pig's liver incubated with dopa for 2 hours. Ordinate: Fluorescence units, Abscissa: Number of tube. Tube 3—4, catechol acetic acid (R = 0.78), tube 7—8, catechol acetaldehyde (R = 0.35), tube 13—15, dopamine (R = 0.18), tube 18—20, dopa (R = 0.13).

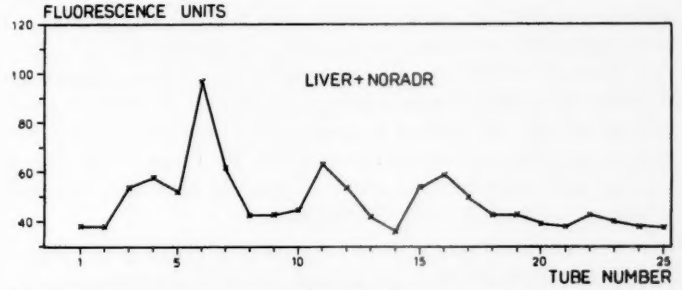

Fig. 2. Chromatogram on starch column of extract of guinea-pigs' liver incubated with noradrenaline for 2 hours. Ordinate: Fluorescence units, Abscissa: Number of tube. Fractions discussed in text.

2. *Noradrenaline.* The type of curve obtained from noradrenaline incubated with liver tissue shows different characteristics as illustrated by Figure 2.

A small peak at tube 31 (R = 0.095) probably corresponded to a small amount of residual noradrenaline. Only a small quantity of activity at the site of catechol acetic acid is present, while the adjacent peak at tube 6 probably corresponds to catechol glycolic acid. The catechol fraction appearing in tubes 15—17 has been

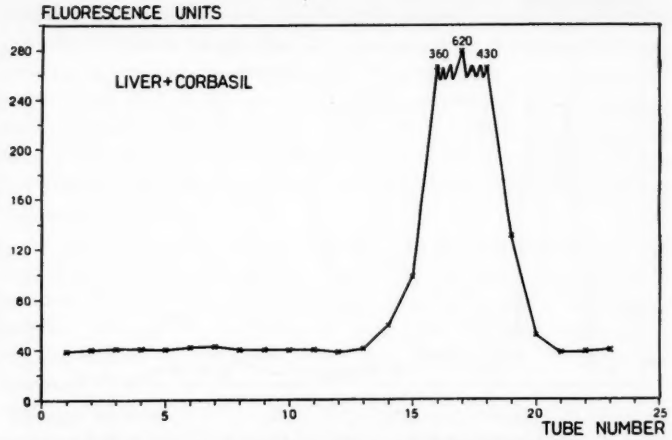

Fig. 3. Chromatogram on starch column of extract of guinea-pig's liver incubated with Corbasil (3 : 4, dihydroxynorephedrine) 2 hours. Ordinate: Fluorescence units, Abscissa: Number of tube.

identified by its biological action and its trihydroxyindol fluorescence reaction as noradrenaline. The peak at tube 11 has not been identified, but may be catechol glycolaldehyde.

3. *Corbasil* (*Cobefrine*). The catechols derived from incubation of liver tissue with Corbasil showed no definite fluorescent products up to tube 14. Tubes 14—20 showed a broad band of activity with a maximum appearing in tubes 16—18. From the biological activity and colorimetrical assay it appears that the catechols consist of unchanged Corbasil (Fig. 3).

Comment.

The location and/or biological activity of the four fluorescence bands occurring on chromatography after incubation of liver tissue with dopa suggest that they are due to unchanged dopa ($R = 0.13$), dopamine ($R = 0.18$), catechol acetaldehyde ($R = 0.35$) and catechol acetic acid ($R = 0.78$). This would be expected since guinea-pig's liver is known to contain l-dopa decarboxylase (Holtz, Heise and Luedtke, 1938) and amine oxidase, which is held to cause the inactivation of adrenaline perfused through the rabbit's liver (Lund, 1951). The catechol acetaldehyde formed by oxidative deamination of dopamine is liable to become further

oxidized to catechol acetic acid by aldehyde oxidases in the tissue (HOLTZ and KRONEBERG, 1950). In the case of noradrenaline the corresponding compounds would be catechol glycolaldehyde and catechol glycolic acid. The occurrence of noradrenaline at an R-value of 0.19, identified by biological assay, may be attributed to the fact that the extract was made with trichloracetic acid which combines with noradrenaline to give a fast-moving compound in paper chromatography (EULER, 1950; SHEPHERD and WEST, 1952).

In the case of incubation of Corbasil with liver tissue no products indicating an effect of amine oxidase were revealed which is in conformity with the fact that Corbasil is not attacked by this enzyme. This finding adds further biological significance to the observation that Corbasil as well as adrenaline and noradrenaline are inactivated in the body to more than 95 per cent, only 3—4 per cent appearing in urine. Since noradrenaline is inactivated much more readily than Corbasil in the liver, the result strongly suggests that another mechanism is responsible for a great part of the inactivation of this substance in the body (EULER and ZETTERSTRÖM, 1955).

While large amounts of catechol acetic acid are found in normal urine the corresponding compound formed by noradrenaline is not observed with certainty. This suggests that dopa is decarboxylated and deaminated in large amounts normally in the body, as found to be the case with liver tissue (EULER, EULER and FLODING, 1955).

Summary.

After incubation of dopa with liver tissue, dopamine, catechol acetaldehyde and catechol acetic acid can be demonstrated by chromatographic separation and formation of fluorescent condensation products with ethylene diamine.

Noradrenaline during the same conditions gives presumably the corresponding hydroxy compounds (catechol glycolic acid and catechol glycolaldehyde).

Corbasil (3 : 4, dihydroxynorephedrine), when incubated with liver, forms no products of similar kind indicating that it is not attacked by amine oxidase.

The presence of large amounts of catechol acetic acid in normal urine suggests that this is formed by decarboxylation, deamination, and subsequent oxidation from dopa.

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A Fluorimetric Micromethod for Differential Estimation of Adrenaline and Noradrenaline.

By

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The iodine method of EULER and HAMBERG (1949) permits the estimation of adrenaline and noradrenaline in mixtures by the different oxidation velocities at different pH values. The method depends on the formation of red oxidation products, adrenochrome and noradrenochrome, partly in conjunction with their iodo-derivatives (EHRLÉN, 1948). By using a wave-length where light absorption is equal for both adrenochrome and iodo-adrenochrome the result is independent of the varying proportions of the latter (EULER and HAMBERG, 1949). The sensitivity of the method, however, only allows determination of amounts down to about 10 μg.

More sensitive methods have been described by EHRLÉN (1948), LUND (1949) and by WEIL-MALHERBE and BONE (1953), which all depend on the formation of fluorescent products. EHRLÉN used ferricyanide as oxidant for the transformation of adrenaline to adrenochrome. The strongly fluorescent compound formed by addition of alkali is very unstable in the presence of oxygen and therefore unsuitable for quantitative estimations unless oxygen is removed. EHRLÉN showed that by addition of ascorbic acid further oxidation could be prevented, whereby the product became stable and could be used for quantitative estimation purposes. He was also the first to identify the fluorescent compound as 1-methyl-3,5,6 trihydroxyindole. This was subsequently verified independently by LUND (1949) and by FISCHER (1949) who

also isolated the fluorescent compound, called adrenolutine by LUND.

LUND (1950) used oxidation with manganese dioxide at pH 3 and 6.5 for differential estimation of adrenaline and noradrenaline in a mixture and measured fluorescence after stabilization of the product according to EHRLÉN.

The method of WEIL-MALHERBE and BONE (1953) is based on the formation of stable fluorescent condensation products of catechol derivatives and ethylene diamine as described by NATELSON et al. (1949). By utilizing the different wave-length maxima of the emitted light obtained with adrenaline and noradrenaline WEIL-MALHERBE and BONE were also able to estimate them differentially in a mixture. Modifications of this method have since been reported by PERSKY and ROSTON (1953) and by MANGER et al. (1953).

Since various catechol derivatives other than adrenaline and noradrenaline usually occur in biological material and give the reaction with ethylene diamine, this method and modifications based on this principle are often unsuitable for estimation of adrenaline and noradrenaline in such material.

Below is described a rapid and sensitive method for fluorimetric estimation of adrenaline and noradrenaline in a mixture.

Experimental.

1. *The Fluorometer.* The fluorescence unit was made by A.B. Svenska Mätapparater, Stockholm, essentially after the principles given by WEIL-MALHERBE and BONE (1953) and connected to the recording system of a Beckman spectrophotometer. Silica cuvettes of the dimensions 23 × 35 × 10 mm were used (WEIL-MALHERBE and BONE 1953). The exciting light from the high pressure mercury vapour lamp (type MBL/D, British Thomson-Houston Co, Ltd.) passed through a condensing lens system and was directed by a microscope mirror through the primary filter and the clear bottom of the cuvette. The emitted light passed through the secondary filter before the photo cell window at the side of the cuvette.

As reference standard quinine sulfate solutions in 0.1 N-H_2SO_4 were used. The fluorescence of the adrenaline and noradrenaline standards were measured in terms of the quinine fluorescence in

order to check the maximum figures for adrenaline when the reaction was carried out at pH 3.5 and 6.0, and for noradrenaline at pH 6.0. All solutions were made up to 10 ml. The cuvettes were filled above a 5 ml mark on the holder. No influence on the fluorescence intensity was observed with different levels of filling above the 5 ml mark. Adrenaline and noradrenaline figures are given in terms of the hydrochlorides.

2. *Oxidation with iodine.* When adrenaline and noradrenaline were present in similar amounts satisfactory results were obtained by oxidation with iodine at pH 4 and 6 and transformation of the coloured compounds into the fluorescent substances. By using suitable primary and secondary filters a ratio of about 1 was obtained for the relative fluorescence intensity of adrenaline: noradrenaline. Similar observations were made by PEKKARINEN (1954). The fading time for the extract blank containing noradrenaline is of the order of 20 minutes as in Lund's technique, which is sometimes inconveniently long. This procedure was therefore abandoned.

3. *Oxidation with ferricyanide.* Ferricyanide treatment according to EHRLÉN for 2—5 minutes at pH 6 gave maximal fluorimetric readings for both adrenaline and noradrenaline. With Schott BG 12 as primary filter and Chance OY 4 as secondary filter the ratio of the fluorescence intensities for equal concentrations of adrenaline and noradrenaline as hydrochlorides was 1.6.

In order to arrive at a differential method for adrenaline and noradrenaline in a mixture the formation of the fluorescent substance was also studied at pH 3—4. However, at this pH the development of adrenochrome and noradrenochrome with ferricyanide was very slow, although a marked difference between adrenaline and noradrenaline was noted. In order to increase the oxidation speed zinc sulfate was added. In this way complete oxidation of adrenaline was achieved in 3 minutes while noradrenaline only gave a small fluorescence. This reaction is apparently of a different kind than that reported by FISCHER et al. (1950) according to whom zinc and aluminium salts transform adrenochrome into the fluorescent compound. In our case the addition of zinc salt expedited the formation of adrenochrome as was confirmed by photometer readings at 529 mμ. The adrenochrome colour intensity remained constant for the 15 minutes during which it was followed.

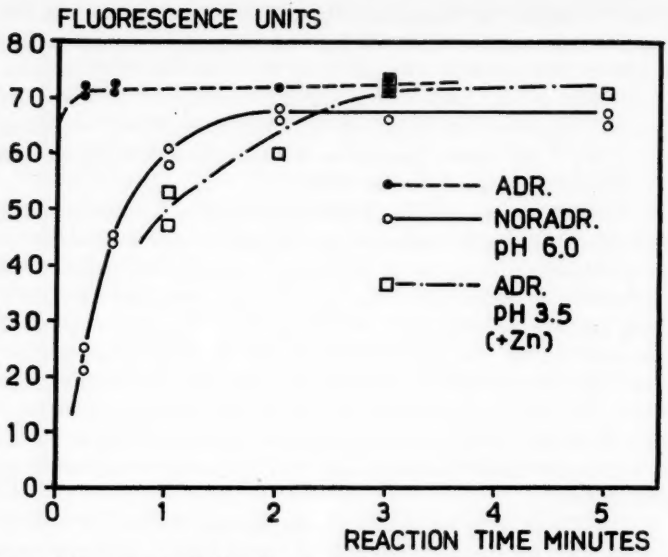

Fig. 1. Influence of oxidation time upon fluorescence intensity for adrenaline and noradrenaline at pH 3.5 ($K_3Fe(CN)_6 + ZnSO_4$) and pH 6.0 ($K_3Fe(CN)_6$). (Read after 10 min.)

4. *Oxidation of adrenaline and noradrenaline at pH 3.5.*

In the presence of potassium ferricyanide and zinc sulfate adrenaline is completely oxidized in 3 minutes, giving maximal fluorescence. During the same time less than 5 per cent of the noradrenaline is oxidized.

5. *Influence of various factors on the fluorescence reaction.*

Several details in the procedure have been systematically investigated in order to obtain optimal reaction conditions. Furthermore the stability of the blank and the fluorescent product have been followed.

a. *Influence of oxidation time.*

Fig. 1 shows the relationship between reaction time and fluorescence intensity for standard solutions of adrenaline and noradrenaline at pH 3.5 and 6.0, using the amounts of reagents described under 6. From the figure it is seen that complete oxidation of both adrenaline and noradrenaline at pH 6.0 is obtained in 2 minutes, and in 3 minutes for adrenaline at pH 3.5 in the presence of zinc sulfate.

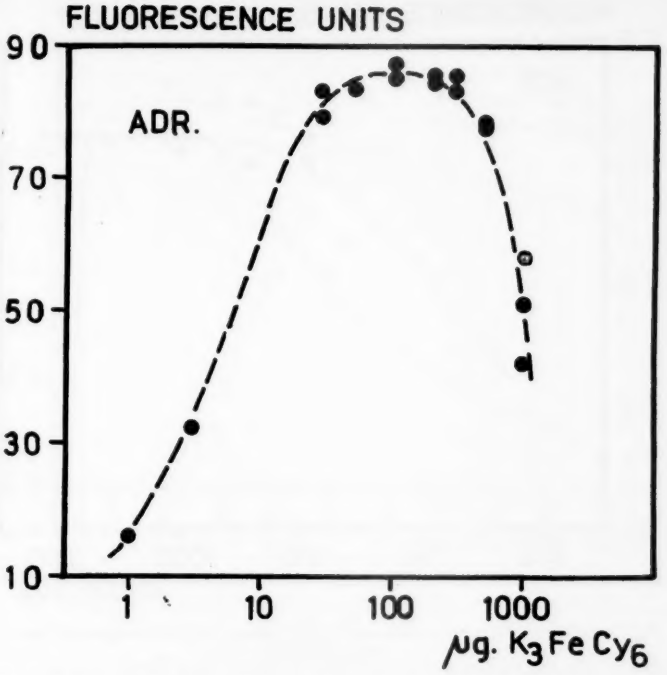

Fig. 2. Influence of various amounts of potassium ferricyanide on the fluorescence reaction with adrenaline.

b. *Effect of varying concentrations of oxidants.*

The amounts of potassium ferricyanide and zinc sulfate have been varied in order to find the optimal concentrations. Figs. 2 and 3 show the results of these experiments for adrenaline. Maximal fluorescence readings are obtained with 0.1 ml 0.25 % $K_3Fe(CN)_6$ and 0.1 ml 0.5 % Zn SO_4 which have been used in the final procedure. Similar results were obtained with noradrenaline.

c. *Effect of varying amounts of ascorbic acid.*

The amount of ascorbic acid added for the purpose of stabilizing the fluorescent products was found to be of importance as already observed by EHRLÉN and by LUND. The maximal fluorescence was obtained in a fairly narrow concentration region of 1—2 mg

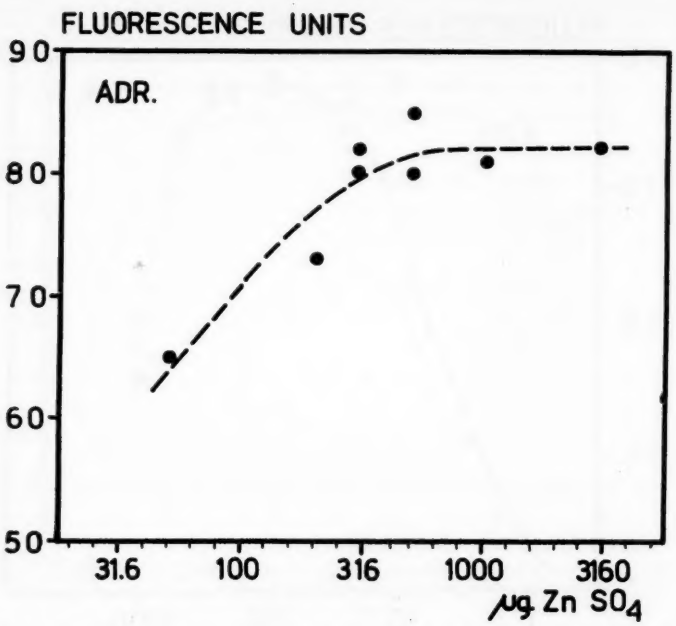

Fig. 3. Influence of various amounts of zinc sulfate on the fluorescence reaction with adrenaline.

ascorbic acid. With larger amounts a quenching effect was noted, especially for noradrenaline. (Fig. 4.)

d. *Stability of blank.*

The fluorescence of the reagent blank has been followed with and without zinc sulfate and with buffer solutions at pH 3.5 and 6.0 and was found to remain approximately constant over a period of one hour.

The differences in the blank fluorescence with and without zinc sulfate and with buffer solutions of different pH values were negligible for most purposes.

e. *Preparing of extract blank.*

An extract and reagent blank were prepared by allowing the fluorescent product to fade (LUND, 1950). By adding the alkali without the ascorbic acid the fading is complete in 1—2 minutes both for adrenaline and noradrenaline. (Fig. 5.)

f. *Stability of fluorescent product.*

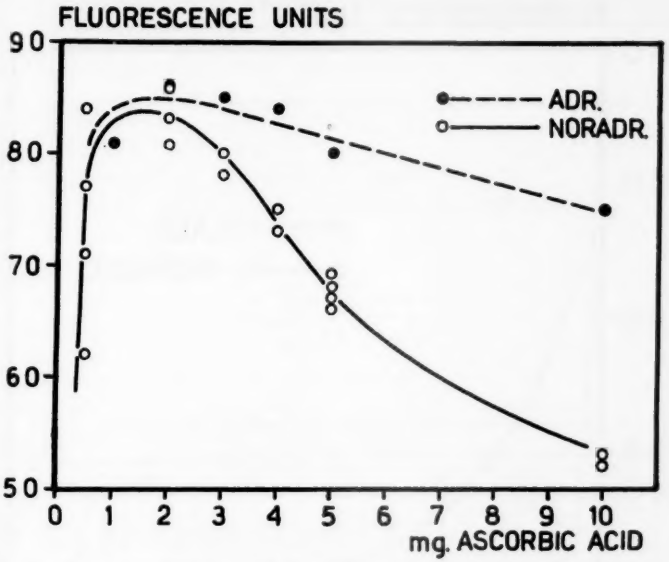

Fig. 4. Effect of various amounts of ascorbic acid on the fluorescence intensity.

EHRLÉN (1948) and LUND (1950) have reported that the fluorescence after addition of ascorbic acid is approximately stable for about one hour. With the present technique we have studied the stability for one hour, using oxidation times of 2 and 3 minutes respectively at pH 6.0 and 3.5 with standards solutions of adrenaline and noradrenaline and with amounts of reagents described under 6. The results are given in Fig. 6.

6. *Fluorimetric estimation of adrenaline and noradrenaline in a mixture.*

On the basis of the observations reported above the following method was adopted.

a. *Reagents.*

The following reagents are used:

Buffer solutions	1 M acetic acid-sodium acetate mixtures of pH 3.5 and 6.0.
Potassium ferricyanide .	0.25 per cent solution
Zinc sulfate	0.5 » » »
Sodium hydroxide	20 » » »

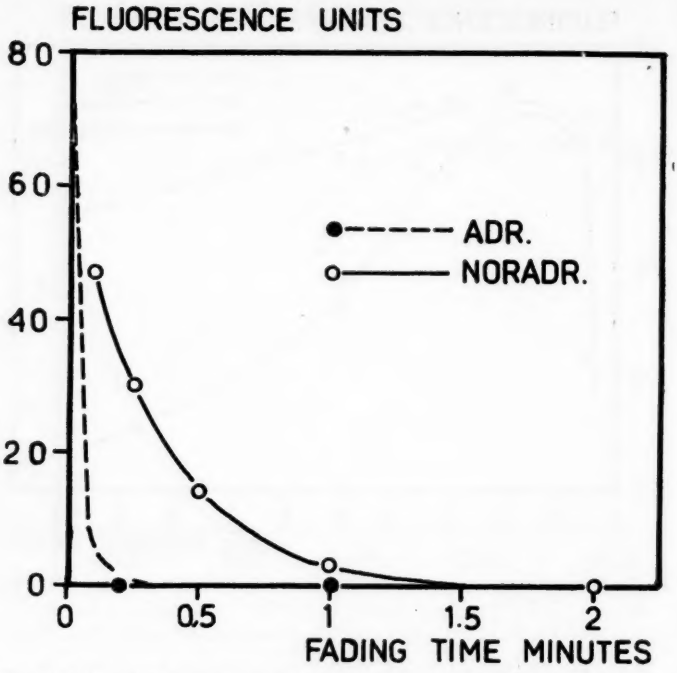

Fig. 5. Fading of fluorescence on adding strong alkali without ascorbic acid in the presence of potassium ferricyanide.

Ascorbic acid ..... 2 per cent solution
Combined reagent ..... 9 ml of the sodium hydroxide solution is mixed immediately before use with 1 ml ascorbic acid solution, prepared on the same day.

For the estimation procedure using the fluorimeter arrangement described above 0.02—0.5 μg adrenaline and/or noradrenaline were used. The sum of adrenaline and noradrenaline is determined at pH 6.0 and adrenaline plus 2—4 per cent of the noradrenaline at pH 3.5.

The maximal fluorescence figures for adrenaline are the same within the limits of error when the reaction is carried out with zinc sulfate at pH 3.5 and without zinc at pH 6.0.

b. *Estimation of adrenaline plus noradrenaline.*

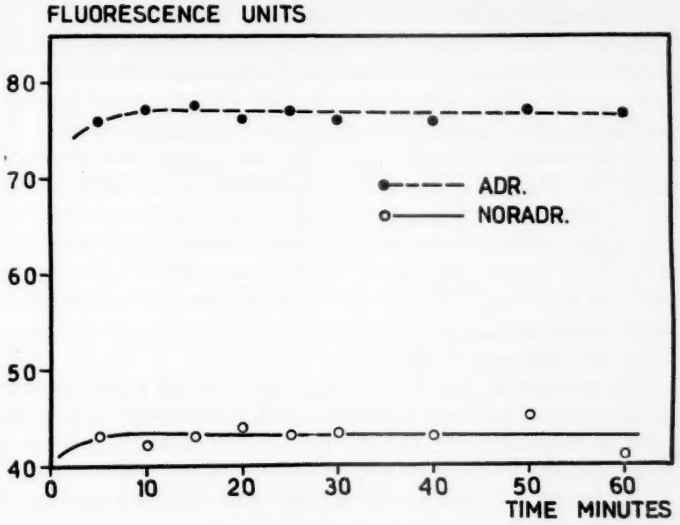

Fig. 6. Stability of fluorescence products of adrenaline and noradrenaline.

A suitable quantity of a sample containing catechol amines is placed in a calibrated 10 ml stoppered glass cylinder, and to this is added 1 ml 1 M-acetate buffer of pH 6.0 and 0.1 ml 0.25 per cent potassium ferricyanide. After 2 minutes 1 ml of a mixture of 9 ml 20 per cent sodium hydroxide and 1 ml 2 per cent ascorbic acid is added and the cylinder filled with distilled water to the 10 ml mark. After thorough mixing the contents are transferred to the silica cuvettes and the fluorescence read after 5—60 minutes against a blank and standard. An extract blank is prepared by treating the sample with ferricyanide as above and adding 0.9 ml 20 % sodium hydroxide and leaving it for 2 minutes. After this time the specific fluorescence has faded. In the presence of ferricyanide the fading of noradrenolutine is much more rapid than with the procedure of LUND (1950) which requires 20—25 minutes. A linear relationship holds between the concentration of the catechol amines and the light transmission reading.

If the sample contains a mixture of adrenaline and noradrenaline the reading at pH 6 is used together with the reading at pH 3.5 as described below.

c. *Estimation of adrenaline.*

To the catechol amines is added 1 ml 1 M acetate buffer of pH 3.5, 0.1 ml 0.25 per cent potassium ferricyanide and 0.1 ml 0.5 per cent zinc sulfate. After 3 minutes 1 ml of the mixture of ascorbic acid and sodium hydroxide is added, the volume filled up to 10 ml and the fluorescence read against the same blank and standard as above. When the amounts of noradrenaline are 50 % or less of the total catechol amines, the pH 3.5 reading gives adrenaline alone for practical purposes, the noradrenaline contributing less than 3 p.c. to the total figure. If the noradrenaline is more than 50 % of the total catechols, the exact amounts are calculated as described below.

d. *Calculations of results.*

The relation of the fluorescence intensities for equal amounts of the hydrochlorides of adrenaline and noradrenaline during the present conditions was 1.6 (= m) and the relative amount of noradrenaline oxidized at pH 3.5 (3 min.) was 4 per cent (= n).

If adrenaline is used as standard and set at 100 with the reagent blank at zero the following relations hold:

A = fluorimeter reading at pH 6.0
B = » » » pH 3.5
F = » » » of faded extract
a = μg standard used (per 10 ml reaction volume).

Then noradrenaline $= y = \frac{a(A - B) \cdot m}{100 - n}$ and adrenaline $= x = \frac{a(B - F)}{100} - \frac{n \cdot y}{100 \cdot m}$.

7. *Application of method to known mixtures of adrenaline and noradrenaline.*

Varying mixtures of adrenaline and noradrenaline were analyzed with the following results (Table I).

8. *Specificity.* As concluded on theoretical grounds by Bu'Lock and Harley-Mason (1953) and observed independently by Lund, only those catechol amines which possess a hydroxy-group at the β-carbon (next to the ring) and hydrogen at the α-carbon in the side chain are able to form fluorescent compounds from the corresponding chromes. Thus for instance, isopropylnoradrenaline (Isuprel, Aludrin) gives a strong fluorescence, while dopamine (in contradistinction to its behaviour with the ethylene diamine reaction), and 3 : 4, dihydroxynorephedrine (Corbasil, Cobefrine)

Table I.

Estimation of adrenaline and noradrenaline in mixtures of known solutions.

Sample No.	Content		Found	
	Adrenaline µg	Noradrenaline µg	Adrenaline µg	Noradrenaline µg
1	0.02	0.18	0.022	0.183
2	0.06	0.14	0.053	0.140
3	0.1	0.1	0.097	0.104
4	0.14	0.06	0.131	0.062
5	0.18	0.02	0.170	0.027

give only a very weak fluorescence. In biological material there is little reason to expect any appreciable amounts of catechol amines with a hydroxy-group in the side chain other than adrenaline and noradrenaline though dopamine has been found in heart extracts and suprarenals (GOODALL, 1951). It should be noted, however, that LOCKETT (1954) recently has claimed that suprarenal extracts may contain small amounts of isopropylnoradrenaline.

Discussion.

The present method possesses certain advantages in comparison with previously described methods of a similar kind.

The oxidation of adrenaline at pH 3.5 is selective for many practical purposes which simplifies calculation of the results. The fading of the blank also occurs very rapidly for noradrenaline. The ratio of fluorescence intensities for noradrenaline and adrenaline is favourable for estimation of small amounts of adrenaline in conjunction with noradrenaline. The specificity of the method for adrenaline and noradrenaline in biological material is high. The method is rapid and easy to perform in any laboratory having access to a suitable fluorimeter.

The application of this method to extracts of tissues and urine will be described in a following paper.

Summary.

EHRLÉN's method for the fluorimetric determination of adrenaline has been modified so as to allow estimation of both adrenaline and noradrenaline in a mixture.

The method is based on differential oxidation of adrenaline and noradrenaline with potassium ferricyanide at pH 3.5 and 6.0.

By addition of zinc sulfate complete oxidation of adrenaline is achieved in a few minutes at pH 3.5 while only some 2—4 per cent of the noradrenaline is oxidized in the same time.

Fading of the fluorescence in alkali without ascorbic acid is completed in 2 minutes.

This work has been supported by the Knut and Alice Wallenberg foundation, which is gratefully acknowledged.

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Fluorimetric Estimation of Noradrenaline and Adrenaline in Urine.

By

U. S. VON EULER and I. FLODING.

The methods for quantitative determination of noradrenaline and adrenaline in urine are generally based on the adsorption of the catechol amines on aluminium hydroxide (EULER, 1948, EULER and HELLNER, 1951) or aluminium oxide (LUND, 1952, PEKKARINEN, 1954). The catechol amines are then estimated either by bioassay (EULER and HELLNER, 1951), or by fluorimetric determination. Using the latter method GOLDENBERG et al. (1954) did not obtain satisfactory results, however, in their attempts to estimate differentially noradrenaline and adrenaline in urine extracts, after oxidation with manganese dioxide according to LUND. The ethylene diamine condensation reaction (NATELSON et al., 1949), on the other hand, depends on the presence of a catechol nucleus; consequently estimations with this method will include various inactive degradation products. It is therefore unsuitable for urine catechol amine estimations. A method for differential estimation of adrenaline and noradrenaline has been described recently by EULER and FLODING (1955). It is based on the formation of the fluorescent trihydroxyindole derivatives of adrenaline and noradrenaline (EHRLÉN, 1948; LUND, 1949; FISCHER, 1949; BU'LOCK and HARLEY-MASON, 1951). The present report describes this method as applied to urine extracts and some results obtained.

Experimental.

The extraction procedure which is described in detail elsewhere (EULER and ORWÉN, 1955) consists briefly in adsorption of the urinary catechol amines on aluminium oxide at pH 8.5 and sub-

sequent elution with 0.3 N oxalic acid. The extract is then subjected to fluorimetric estimation of the trihydroxyindole derivatives obtained by oxidation with potassium ferricyanide, rearrangement with alkali, and stabilization with ascorbic acid (EHRLÉN, 1948). In order to achieve differential formation of the fluorescent products the reaction is carried out at pH 3.5 and 6.0 according to the principle described by EULER and HAMBERG (1949). The oxidation of adrenaline at pH 3.5 is greatly enhanced by the addition of zinc sulfate, which has been found by FISCHER et al. (1950) to also cause a transformation of adrenochrome to its fluorescent product.

The fluorimeter used was constructed according to WEIL-MALHERBE and BONE (1953) and used in conjunction with the recording system of a Beckman DU spectrophotometer. For the primary filter a Schott BG12 was used and for the secondary filter, a Chance OY4.

The fluorimetric estimation is carried out at pH 3.5 and pH 6.0 using an amount of urine extract corresponding to about 0.05—0.25 μg noradrenaline, which is contained in approximately 1—5 ml urine or 0.1—0.5 ml of the extract.

Procedure.

In a calibrated 10 ml stoppered glass cylinder 1 ml 1 M acetate buffer of pH 6.0 is added to the extract which should have a pH of not less than 4 and little buffer capacity. To this is added 0.1 ml 0.25 % potassium ferricyanide which is allowed to act for 2 minutes. After this time 1 ml of a freshly prepared mixture of 9 ml 20 % sodium hydroxide and 1 ml 2 % ascorbic acid are added. The ascorbic acid should be prepared on the same day or stored in a deep freeze box. The volume is then filled to 10 ml with distilled water. On addition of ascorbic acid the ferricyanide is reduced and the solution becomes colourless. The rearrangement to the fluorescent compounds is completed in less than 5 minutes and the fluorescence determined. In order to remove a slight cloudiness caused by traces of aluminium hydroxide the sample may be centrifuged at 2,000 r.p.m. for 1/2 minute. This leaves a perfectly clear supernatant which is essential for the fluorimetric estimation.

At pH 6.0 both noradrenaline and adrenaline are completely oxidized in 2 minutes. When the reaction is carried out at pH 3.5, adrenaline is completely oxidized to adrenochrome in the presence of zinc sulfate at a time when only a small proportion of the noradrenaline is oxidized. A second estimation is therefore made at pH 3.5. To the extract sample are added 1 ml 1 M acetate buffer of pH 3.5 and 0.1 ml 0.25 % potassium ferricyanide solution. In order to enhance the oxidation 0.1 ml 0.5 % zinc sulfate solution is added. Adrenaline is then oxidized to adrenochrome in 3 minutes while only about 4 p.c. of the noradrenaline is oxidized.

The readings are made against a blank in which the fluorescent product has been allowed to develop and fade by adding all reagents except the stabilizing ascorbic acid. Complete fading of adrenaline and noradrenaline is obtained in less than 2 minutes. The fluorescence of the samples at pH 3.5 and 6.0 is read against a noradrenaline standard set at 100 (or a quinine sulfate reference standard) with the reagent blank set at zero.

Calculation of Results.

Fluorescence reading at pH 6 = A
» » » pH 3.5 = B
» » after fading = F (= extract blank).

If the amount of noradrenaline standard = a, the relation of the intensity of the fluorescence for $\frac{\text{adrenaline}}{\text{noradrenaline}} = m$, and if the percentage noradrenaline oxidized at pH 3.5 in 3 min. = n, the amounts present in the samples are

$$\text{Noradrenaline} = y = \frac{a\ (A - B)}{100 - n}$$

$$\text{Adrenaline} = x = \frac{a\ (B - F)}{100 \cdot m} - \frac{n \cdot y}{100 \cdot m}$$

The amount of noradrenaline standard has generally been 0.1—0.2 μg with the fluorimeter used in this investigation. The values of m has to be determined for each set of filters; in the present work its numerical value was 1.6. The figure used for n was 4.

Table I.

Comparison of fluorimetric estimation and biological assay in urine extracts including four cases of verified phaeochromocytoma (μg per 100 ml urine).

Sample	Biological assay		Fluorimetric estimation	
	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
E.	2.3	2.6	2.3	3.5
E. K.	0.39	2.3	0.59	1.6
R. A.	0.83	5.7	2.1	6.8
E. Å.	0.16	1.9	0.57	2.1
1	0.24	0.38	0.57	0.99
2	0.21	1.3	0.56	1.5
3	0.24	0.88	0.88	2.5
W.	0.41	1.8	0.42	3.1
P.	0.04	0.96	0.21	0.94
O.	0.15	0.50	0.36	0.66
S.-L.	0.65	0.80	0.96	1.7
E-m.	0.94	3.2	1.0	2.0
F. 1	0.62	2.2	1.2	2.1
F. 2	0.58	1.8	1.0	1.5
Å.	0.47	2.6	0.70	2.8
A-m[1]	0.09	5.0	0.22	4.2
A-m[1]	0.04	4.2	0.20	4.2
S. F.[2]	47	56	49	39
K. F.[2]	0.4	60	4.4	61
L. L.[2]	—	80	1.8	78
M. W.[2]	0.41	100	5.3	82

[1] Adrenalectomized patient.
[2] Phaeochromocytoma.

Comparison of Biologic and Fluorimetric Estimations.

A series of estimations of adrenaline and noradrenaline in urine extracts was made by the biologic and fluorimetric methods (Table I). The first four and the last four samples in Table I, used for fluorimetric estimations, were prepared by elution with 0.25 N sulfuric acid and the rest made by elution with 0.3 N oxalic acid, while either sulfuric or oxalic acid was used for the extracts in the biological assay.

In the course of the experiments it was observed that the discrepancy in the adrenaline figures was reduced when oxalic acid was used for the elution instead of sulfuric acid. This may account for the larger deviation in the adrenaline figures in the four cases of phaeochromocytoma.

The deviations between the two methods vary between moderate limits. In several of the samples the agreement is very good while in others the difference is well outside possible errors in the estimation techniques, indicating a real difference. The cause of this cannot at present be stated.

In the four cases of phaeochromocytoma the good agreement in general between the fluorimetric and the biologic methods clearly indicate the usefulness of the former method for clinical laboratory analysis of urine from suspect cases as noted by other authors.

The two samples from an adrenalectomized patient (A-m) show low relative adrenaline figures with both methods. Generally the fluorimetric figures are higher than the biologic ones, though the difference in the noradrenaline figures is usually small.

Summary.

A method for fluorimetric estimation of adrenaline and noradrenaline in urine is described. The urine extracts are prepared by adsorption on aluminium oxide at pH 8.5 and elution with 0.3 N oxalic acid.

The catechol amines in the extracts are oxidized to adrenochrome and noradrenochrome at pH 3.5 and 6.0 with potassium ferricyanide. The reaction is enhanced by zinc sulfate at pH 3.5. After rearrangement with strong alkali and stabilization of the fluorescent product with ascorbic acid the fluorescence intensities are measured.

At pH 6.0 the sum of adrenaline and noradrenaline is determined and at pH 3.5 adrenaline plus about 4 per cent of the noradrenaline. From these data and the relative fluorescence of adrenaline and noradrenaline the amounts of adrenaline and noradrenaline can be calculated.

This work has been supported by a grant from the Knut and Alice Wallenberg Foundation which is gratefully acknowledged.

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Studies on the Release of the Adrenergic Neurotransmitter from the Perfused Ox Spleen.

I. Action of acids.

By

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Although it is now firmly established that the adrenergic nerve transmitter is identical with levo-noradrenaline, very little is known about the exact localization and the mode of storage of the neurotransmitter in the postganglionic fibre terminals or the factors governing its release.

In continuation of previous work in this laboratory on the physiology of the adrenergic transmitter we have attempted to study some factors which may be operative in the release of the active substance from the nerve terminals.

Material and methods.

The ox spleen was chosen for the perfusion experiments on account of its high content of noradrenaline. Less than 1 hour after the slaughter the organ was brought to the laboratory. Cannulas were inserted in the main artery and vein and about one half of the spleen clamped off. For the perfusion, Tyrode solution with 15 % Macrodex at 37°C aerated with 6.5 % CO_2 in O_2 was used. The organ was placed in a container heated with an electric bulb. Perfusion was maintained by hydrostatic pressure and adjusted to about 10 ml per min. The perfused fluid was collected for periods of usually 10—20 minutes in vessels containing 2 ml 2 N sulfuric acid.

The fluid was subjected to extraction and adsorption of the catechol amines on aluminium hydroxide or on aluminium oxide according to EULER and ORWÉN (1955). After elution with sulfuric acid noradrenaline was determined biologically in the extract on the cat's blood pressure. In some instances adrenaline was also estimated by using the fowl rectal caecum according to EULER (1949).

The substances whose action on the release of the transmitter was to be studied were added continuously to the Tyrode solution by means of a continuous injection device. This was adjusted to give about 1/10 the volume of Tyrode solution flowing through the preparation. Dilution of perfusion fluid by 1/10 with distilled water had no effect on the catechol amine release. The first few hundred ml of perfusion fluid, which were blood-stained, were usually discarded. pH was measured electrometrically in the inflowing and outflowing solution.

Results.

I. *"Spontaneous" release during perfusion with Tyrode solution.*

In order to check whether inactivation of the released transmitter is likely to occur noradrenaline was added to the perfusion fluid in a concentration of about 1 μg per 10 ml and the amount recovered in the perfusate measured. A loss was encountered which amounted to about 50 p.c. of the added quantity. Circulating noradrenaline therefore seems to be partly destroyed in the spleen. In the presence of Marsilid 1 : 10,000 in the Tyrode the loss was greatly reduced, however.

In four experiments the release during continuous perfusion for a few hours with Tyrode solution alone was determined.

Table I.

Spontaneous release of noradrenaline from ox spleen perfused with Macrodex-Tyrode solution. Weight of spleen 500 g.

Time min.	30	10	30	30	20
Volume perfused	175	150	150	170	125
Noradr. μg/100 ml in perfusate.	0.25	< 0.25	< 0.25	0.5	0.25
Noradr. μg/kg/min.	0.029	< 0.075	< 0.025	0.057	0.021

In Table I the release during one experiment of this kind is tabulated. As seen in the Table the maximal release was 0.06 μg per kg per min. In three other experiments the release during a period of 2—3 hours did not exceed 0.05 μg/kg/min. However, when the perfusate was collected immediately after starting the perfusion, higher figures where usually found in the first samples.

During the control periods before the addition of the various solutions, the "spontaneous" release seldom exceeded 0.1 μg/kg/min.

In a few experiments it was noted that considerably larger amounts of noradrenaline were continuously released during perfusion with Macrodex-Tyrode solution alone. It was eventually found that boiling the rubber tubings of the perfusion apparatus before each experiment prevented this effect which was ascribed to toxic products caused by bacterial activity.

II. *Perfusion with acids.*

a) *Ascorbic acid.* Ascorbic acid or ascorbic acid—sodium ascorbate mixtures were added to a final concentration of 0.1 M in six experiments and the amounts of noradrenaline in the perfusate measured. Table II shows the release before, during, and after infusion of ascorbic acid.

Table II.

Effect of ascorbic acid on release of noradrenaline from the perfused ox spleen. Weight of spleen 550 g.

Time min.	20	25	20	20	20	20	20
Volume perfused, ml.	200	260	200	220	200	230	190
Ascorbic acid in perfusion fluid	—	—	0.1 M	0.1 M	0.1 M	—	—
Noradr. μg/kg/min.	0.09	0.09	0.73	0.40	0.73	0.21	0.65
pH of outflowing perfusion fluid	7.1		3.7		3.6	5.5	6.5

From the Table it is seen that this concentration causes a considerable release of noradrenaline, lasting even after the end of the infusion when pH in the perfusate had risen above 5.5.

In other experiments the ascorbic acid was partially neutralised with sodium hydroxide to different degrees of acidity. Fig. 1 shows the effect of an infusion of sodium ascorbate at pH 7.0 (0.1 M in the perfusion fluid) and at pH 3.0. No definite effect

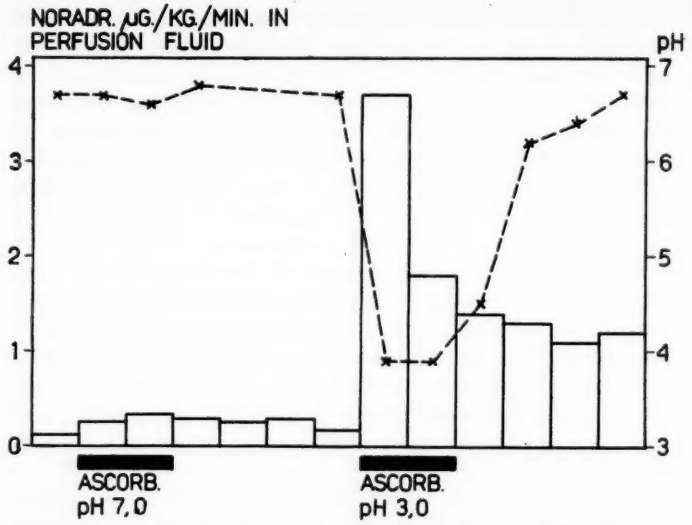

Fig. 1. Release of noradrenaline from the isolated perfused ox spleen on infusion of sodium ascorbate at pH 7.0 and partially neutralized ascorbic acid at pH 3.0. Concentration of ascorbic acid in the perfusate 0.1 M. Columns indicate noradrenaline µg/kg/min. (left ordinate), broken line shows pH values in the perfusate (right ordinate). Each column represents a perfusion time of 20 minutes.

was observed with the neutral solution, while the partly neutralized ascorbic acid had a strong effect on the release, lasting for several periods of 20 min. and still persisting when the pH of the perfusate had reached 6.7—6.8.

Inconsistent effects were observed with ascorbic acid—ascorbate solutions of pH 6.7 (causing a fall of pH in the perfusate from 6.9 to 6.8). A considerable release was noted, however, with ascorbic acid—ascorbate solutions of pH 5.0, which caused a drop in pH in the perfusate from 6.8 to 5.9.

b) *Lactic acid, citric acid.* Solutions of 1 M lactic acid and citric acid partly neutralized with sodium hydroxide to pH 3.7 and 4.8 were used in three experiments. The extra release of noradrenaline from the spleen was comparable to the amounts liberated by ascorbic acid at the same pH. A considerable increase was recorded even during the periods following the actual perfusion, when the pH had already risen to 6.3—6.6. The results are included in Fig. 2.

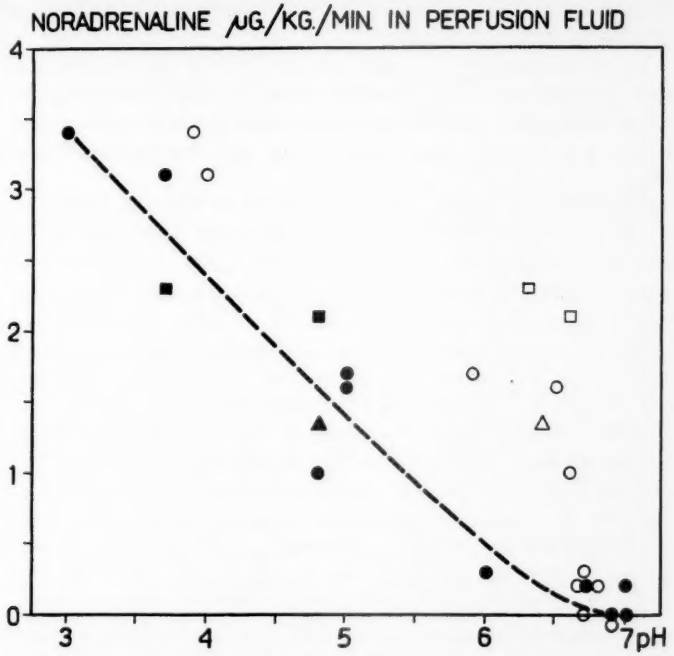

Fig. 2. Release of noradrenaline from the isolated perfused ox spleen on infusion of ascorbic acid/ascorbate (circles) lactic acid/lactate (squares) and citric acid/citrate (triangles) to a concentration of 0.1 M in the perfusion fluid. Full symbols and broken line indicate pH values of infused solution at the end of the infusion period, open symbols refer to pH in the perfusate. An increased release is noted when pH of the perfusate is 6.6 or lower. Ordinate μg noradrenaline released per kg per minute.

Comment.

A change in the reaction of the perfusing fluid to the acid side using naturally occurring acids such as ascorbic, lactic, and citric acid, caused a release of the adrenergic transmitter from the spleen. From the series with ascorbic acid it emerges that the effect is dependent on the actual acidity of the solution. It is notable that an extra release of transmitter could be observed even when the acidity of the added solution was pH 6, causing a fall of pH in the perfusate from 6.9 to 6.6 only. Changes of this order might possibly occur under various conditions in vivo, for instance

in hypoxic and asphyxial conditions and during inadequate circulation such as in shock.

The importance of the acidity for the linkage of adrenaline and noradrenaline to lec thin has been demonstrated by NORLANDER (1950) who found that the amounts of the catechol amines combining with lecithin rose steeply when pH was increased from about 4.5 to pH 7—7.5.

Attention has recently been drawn to the specific structures of the chromaffin cells of the adrenal medulla, which contain the catechol amines (HILLARP and NILSON 1953, BLASCHKO and WELCH 1953). It has later been shown (HILLARP and NILSON 1954) that these granules retain their contents in isotonic, neutral solutions, but readily lose their catechol amines during treatment with acids, in hyposmotic solution and during other conditions involving a disturbance of their natural surrounding milieu.

Some arguments in favour of the concept of specific structures for the storage of catechol amines in nerves has recently been presented (EULER, 1954). If this were the case, one would expect that the noradrenaline containing structures would behave in a similar fashion in the chromaffin cell and in the postganglionic neuron. The results presented here give some support to this idea.

Whatever relations, if any, may exist between release of catechol amines by change of acidity and nerve stimulation can only be a subject of speculation so far, but it would appear that even a very moderate local shift in the reaction in the postulated "granule equivalents" may be sufficient to cause changes in their membrane structures conducive to a release of their contents.

It should also be recalled that special structures have been observed repeatedly in the histological pictures of postganglionic sympathetic nerve terminals giving these the appearance of a bead-string (HILLARP, 1946; GARVEN and GAIRNS, 1952; WEDDELL and PALLIE, 1954).

Summary.

Small amounts of noradrenaline, 0.02—0.1 μg/kg/min., are released "spontaneously" from the ox spleen when perfused with Tyrode solution.

Addition of acids (ascorbic, lactic, citric acid) to the perfusing fluid causes a large increase in the amount of noradrenaline released, up to 4 μg/kg/min. The amounts released are correlated to

the acidity of the perfusing fluid. A definite increase in the release has been observed even when the drop in pH has only been from 6.9 to 6.6 in the perfusate.

Some implications of the findings and their bearing on the storage and release problem of the adrenergic transmitter substance are discussed.

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Studies on the Release of the Adrenergic Neurotransmitter in the Perfused Ox Spleen.

II. Effects of various membrane active substances.

By

U. S. von EULER and L. STJÄRNE.

In a previous communication it has been shown that addition of acids to the Tyrode solution used for perfusion of the ox spleen causes a release of noradrenaline into the perfusate. The effect was strongly correlated with the pH of the added fluid and of the perfusate. Thus no effect was observed when the pH in the perfusate was 6.7—7.0. However, when the perfusate was at pH 6.5 and lower, a release was observed, which increased with falling pH (ELIASSON, EULER and STJÄRNE, 1955).

Working on the hypothesis that the noradrenaline present in the adrenergic nerves is stored in specific structures which may be sensitive to membrane active substances we have studied the effect of various compounds such as digitonin, saponin, detergents and bile acids added to the perfusing Tyrode solution upon the release of noradrenaline. The effect of variations in the cation content of the Tyrode solution was also studied to some extent. Further the action of some amine oxidase inhibitors has been tested.

Methods.

The ox spleens were brought fresh from the slaughter house and perfused with 37°C Tyrode solution containing 15 % Macrodex, and aerated with 6.5 % CO_2 in O_2. Usually only about half the spleen was used; the remainder being shut off from the circulation by a broad clamp.

The perfusate was collected during 10—20 min. periods and was extracted according to EULER and ORWÉN (1955) and assayed for noradrenaline on the cat's blood pressure.

Substances added to the perfusing fluid were dissolved in Tyrode solution or distilled water and infused near the arterial cannula by means of a continuous slow injection device. The noradrenaline figures are given in terms of the hydrochloride. Since the adrenaline present in the extract did not exceed 20 per cent of the total activity and the adrenaline : noradrenaline activity ratio was about 0.5, on the cat's blood pressure the error in noradrenaline estimation did not exceed 10 p.c. and probably was considerably less in most cases. The substances were infused during 10—20 min. in a volume approximately 1/10 of the perfusate.

Results.

Digitonin. Digitonin caused an increased release of noradrenaline into the perfusate in concentrations above 10^{-5} but had no action in weaker concentrations (Fig. 1). It was also observed that digitonin caused very marked vasoconstriction in concentrations of 1 in 20,000 or stronger. This effect did not occur when weaker concentrations were applied.

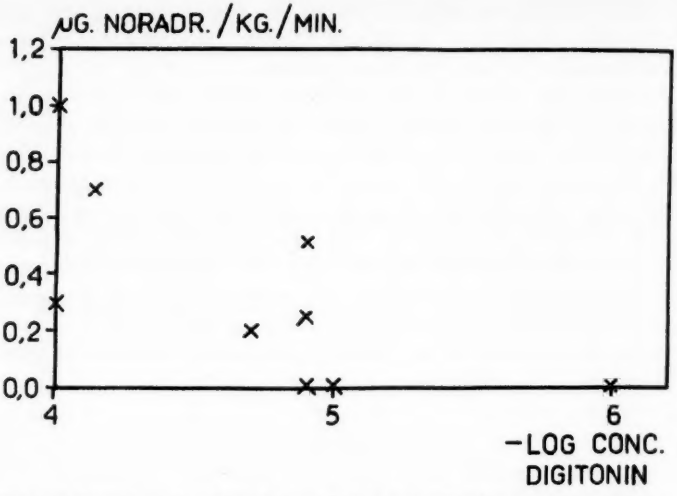
Saponin. In five experiments saponin (Merck) was added to the perfusion fluid in a concentration of about 10^{-4}. In one case only, a slight increase in the noradrenaline release was noted (0.4 μg/kg/min.). Whether or not the increase observed about 1 hour after the perfusion with saponin was due to this substance cannot be ascertained.

Sodium taurocholate. Sodium taurocholate had no effect on the release of noradrenaline from the perfused spleen in concentrations of 10^{-3} and weaker. A slight inconstant effect was noted with 10^{-4}.

Cetyl-pyridinium bromide. No effect was noted with concentrations of 10^{-5} or weaker, while 10^{-4} had a definite effect on the release (0.65—1.5 μg/kg/min.) in 3 out of 4 experiments.

The effect was increased after addition of Marsilid 10^{-4}—10^{-5} to the Tyrode solution, though Marsilid alone in concentration of 10^{-5} had no effect.

Lecithinase. Lecithinase administered as dried snake venom dissolved in water and added to a concentration of 1 : 10,000 had



Release of noradrenaline from the perfused ox spleen during continuous infusion of digitonin in different concentrations.

a moderate but definite effect, the increase being 0.5 µg/kg/min. noradrenaline. Weaker concentrations had no noticeable effect.

Changes in potassium concentration. On increasing the potassium content of the Tyrode solution to double the normal concentration no change occurred in the "basal" release. On increase to 3 or 4 times the normal, K caused, however, a definite increase in the noradrenaline release (1.0 µg/kg/min.).

Effect of nerve stimulation. The increase in the release of noradrenaline as a result of nerve stimulation has been demonstrated repeatedly (PEART, 1949; MANN & WEST, 1950; MIRKIN, 1953). In order to check the responsiveness of the nerves the release was determined in two experiments. A considerable effect (0.8 µg/kg/min.) was observed when a frequency of 100 and a pulse duration of 10 msec. was used.

Amine oxidase inhibitors. Methamphetamine in a concentration of 10^{-5} increased the noradrenaline content in the perfusate to a considerable extent in two experiments (0.6—1.1 µg/kg/min.) and less in three. It was also noticed that the extracts elicited an inhibitory effect on the response of the chicken rectal caecum to catechol amines. A deposition of melanin-like material was ob-

served in the extracts in one of the experiments after addition of methamphetamine.

A small but definite effect was observed after addition of octanol to a concentration of 10^{-4} in two experiments.

Marsilid was added in concentrations 10^{-4}—10^{-5} in 11 experiments. No effect was noted on the "spontaneous" release even in concentrations of 10^{-4}. On the other hand, this concentration of Marsilid seemed to increase the yield when other substances were added, possibly exerting a protective action. The recovery of added noradrenaline was increased even in lower concentrations.

Comment.

A variety of substances have been found to increase the release of noradrenaline from the perfused ox spleen. Digitonin and the detergent cetyl-pyridinium bromide were found to increase the release, while saponin was less active. Digitonin and detergents have previously been shown to release catechol amines from the secretion granules of chromaffin cells (HILLARP and NILSON, 1954). Increased potassium concentration had the same effect.

Amine oxidase inhibitors, such as methamphetamine, octanol and Marsilid when used alone did not regularly increase the release or yield of noradrenaline from the perfused spleen, but did increase the yield after perfusion with other substances. This suggests a protective action on the released transmitter, presumably by inhibition of the inactivating amine oxidase.

The similarity of action by certain membrane active substances on the release of catechol amines from chromaffin cell granules and adrenergic nerves suggests that the transmitter is stored in structures limited by a membrane.

Summary.

Membrane active substances, digitonin, cetyl-pyridinium bromide and potassium ions which cause a release of catechol amines from chromaffin cells also release noradrenaline from adrenergic nerves in the ox spleen perfused with Tyrode solution.

Amine oxidase inhibitors (octanol, Marsilid, and methamphetamine) increased the yield following nerve stimulation and ad-

ministration of substances capable of releasing noradrenaline from the adrenergic nerves.

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